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ERGOSTEROL CONTENT AND ACTIVITIES OF POLYAMINE BIOSYNTHESIS ENZYMES TO CHARACTERIZE MYCORRHIZAL ROOT:SOIL SYSTEMS

by

BRADLEY NEIL JOHNSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

Analyses of ergosterol and activities of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) were examined as estimates of fungal mass and metabolic activity, respectively, in pure cultures of *Hebeloma crustuliniforme*, loamy sand with or without added *H. crustuliniforme*, and roots of *Pinus contorta* seedlings mycorrhizal with *H. crustuliniforme* grown in a loamy sand.

Mycelia at colony perimeters contained 1.7 times more ergosterol and up to 3.6 times more ADC and ODC activity g^{-1} mycelia than those at the core. The regression equation $Y = -5.46 + 0.38(X)$, where Y = mycelia mass (mg) and X = ergosterol mass (μg), accounted for 77% of the variability in colony mass. The ratio of ODC:ADC activity was 21 at the perimeter and 30 at the core; activity ratios may be used to infer maturation state of a fungal colony.

When ^{14}C-labelled mycelia were added to a loamy sand, up to 14% of the associated ergosterol remained as extractable ergosterol after a 22 day incubation; of the remainder, nearly two-thirds was respired as CO_2. Turnover of ergosterol was evident; up to 80% of ^{14}C-labelled ergosterol isolated from *H. crustuliniforme* and added to soil remained as extractable ergosterol after a 42 day incubation and 8% was evolved as CO_2 during incubation.

Ergosterol was compared to chitin as an index of intramatrical mycelial mass. Ergosterol analyses were 1/2 as labor intensive as chitin analyses. Intramatrical mycelia estimates by the two techniques were not correlated ($r = 0.15$) and ergosterol-based estimates were 2 to 5 times greater than with chitin.

With addition of mycorrhizal inoculum to loamy sand, ODC activity mg^{-1} root increased up to 2 fold within 21 weeks of planting while root mass per seedling was not affected. Inoculation increased mycelia mass mg^{-1} root by up to 2 fold but no differences were observed on a total seedling basis until 35 weeks. At this time, inoculated seedlings had 1.7 times greater root mass and 1.3 times more shoot mass. Rhizosphere soil contained 5 times more mycelia and 6 times greater ODC activity than nonrhizosphere soil. Inoculation increased rhizosphere metabolic activity and ergosterol content.

Ergosterol is a sensitive indicator of changes in fungal mass in pure cultures, soils and roots. Estimates of fungal mass by this technique should not be compared to those by other techniques without first determining the extent to which the techniques correlate. Ergosterol analyses of soil extract both biotic and abiotic sources. Thus, background quantities of abiotic ergosterol in soil should be predetermined. Levels of ODC and ADC activity may reflect soil biological activity, but not activities of specific taxa. Ratios of ODC:ADC may provide insight into relative contributions to total soil biological activity among taxa.

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1 INTRODUCTION

Roots of most plants form mycorrhizal associations with one or more species of symbiotic soil fungi. In coniferous plants, mycorrhizae have long since been associated with enhanced plant nutrient acquisition, drought tolerance and pathogen resistance (Marx 1969; Lamb and Richards 1971). The potential commercial benefit has created interest in the manipulation of ectomycorrhizae, often involving the coupling of receptive host seedlings to beneficial mycobionts. The success of such inoculation programs has varied, however, with seedling growth and survival being increased, decreased or not affected by the introduced fungus (Mikola 1973) . This underlines the complexity of mycorrhizae and suggests that root–fungi–soil interactions may be resolved within several hierarchical levels including:

- the landscape level, for example field competence and plant response to inoculation.
- the whole plant, for example effects of mycorrhizae on plant growth and nutrition.
- the root fungus interface, for example ultrastructure, exudation and hormonal relations.
- the plant and fungal genomes, for example microbiont specificity, host susceptability and mycorrhiza effectiveness.

According to principles of hierarchical theory (Webster 1979; Allen *et al.* 1984), biological processes within each level may be assembled into higher levels of organization constituting the ecosystem. Presently, scientists are not able to successfully manage mycorrhizae. This may be due to an inability to make observations on the physiology of ectomycorrhizae which would be meaningful for interpretation of whole plant responses.

With symbiotic fungi and plant roots present as distinct entities, descriptions of the functional niche of each could assist the development of a mechanistic understanding of mycorrhizal root–soil systems. Wiebe (1984) argued for the utility of monitoring microbial processes for developing ecosystem theory and thus, this project focuses on the root–fungus interface as a tripartite system where roots, mycorrhizal fungi and the soil interact.

A system of such biotic, physical and chemical complexity must be examined using quantitative tools which work with precision and accuracy in all components. Successful quantitative indices in mycorrhizal research permit relationships between fungi and roots, among soil microorganisms along roots and in the soil as a growth medium to be described. Furthermore, two quantitative aspects of the system are of interest. First, knowledge of fungal mass in roots is necessary to determine the success of introduced inocula as root colonizers. Second, metabolic activity of roots and soil biota must be estimated in order for influences of fungal inocula and mycorrhizae to be determined.

Morphological techniques have been used by several authors to quantify fungal mass (Marx and Bryan 1975; France *et al.* 1985). Visual determinations of the percentage of root length with sheath development are commonly made, or the proportion of mycorrhizal roots compared to non–mycorrhizal short roots is estimated. Interpretive problems arise from the fact that over 90% of the root may be ensheathed, thereby excluding intramatrical hyphae from enumeration (Shaw *et al.* 1982). Intramatrical and extramatrical hyphae may be present in 1:1 proportions (Hepper 1981), hence the extent of mycorrhiza establishment may be underestimated when morphological techniques are applied.

Hyphal mass in soils and roots may also be quantified by biochemical techniques. West *et al.* (1987) concluded that ergosterol may be a sensitive indicator of changes in soil fungal mass and Soderstrom (1977) used fluorescein diacetate staining to partition active hyphae from inactive. Ergosterol, being a principle sterol of fungi within the Basidiomycotina subphylum (Weete 1974) and being absent in most in plant tissues (Nes 1977), was used by Salmanowicz and Nylund (1988) as an estimate of intramatrical mycelia establishment in *Pinus sylvestris* roots. Their estimates were based on the ergosterol content of 2 to 4 week old *Hebeloma crustuliniforme* colonies. Ontological and environmental influences on ergosterol content have not been described; this will be undertaken in this project.

In addition to estimates of fungal mass, the impact of fungal inocula on soil biotic activity, root biotic activity and plant survival and growth would increase the knowledge and understanding of mycorrhizal associations. Such determinations are not common. Nye and Tinker (1978) suggested that mechanisms which regulate nutrient movement and concentration within plants need to be examined. Recently, hormonal interactions among the mycobiont and host have been reported (Allen *et al* 1982) and the rhizosphere has been shown to differ from bulk soil with respect to enzymology (Reddy *et al.* 1987) and microflora composition (Marschner *et al.* 1987). Nonetheless, biochemical indices of biological activity have not been used extensively to clarify the effects of mycorrhiza establishment on root metabolism and rhizosphere ecology. Activities of the polyamine biosynthesis enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) have been correlated to rates of protein synthesis, DNA replication and tRNA functions in selected plant, microbial and mammalian systems (Pegg and Williams–Ashman 1981; Galston 1983, Walters *et al.* 1985). Activities of these enzymes as potential analogues of metabolic activity in mycorrhizal root:soil systems has not been examined.

The purpose of this project was to develop and apply biochemical techniques to quantify mass/activity relations of mycorrhiza-root soil systems, specifically to determine if analyses of ergosterol and activities of polyamine biosynthesis enzymes can be applied as indices of fungal mass and metabolic activity, respectively. The research program was developed upon the following questions:

1) How, do ergosterol concentrations and activities of polyamine biosynthesis enzymes vary in vegetative mycelia of different maturation states and subject to different media nutritional conditions?

2) Does ergosterol persist in soil material?

3) Do vegetative mycelia of mycorrhizal fungi affect soil metabolic activity in the absence of roots?

4) How does the ergosterol analysis compare to chitin as a predictor of fungal mass in soil or roots?

5) What impact does mycorrhiza establishment have on root metabolism? Does the effect vary with period of root growth or nutritional status of the rooting medium?

6) What impact do mycorrhizal fungi have on soil biotic activity and fungal mass? Does the effect vary over periods of root growth, distances from roots or nutritional status of the rooting medium?

System complexity was progressively increased during this project, starting with pure-cultured mycorrhizal fungi, followed by fungi in soil without roots and fungi in soil with a host plant present. This represents an original contribution to studies of soil ecology in general and mycorrhiza symbiosis in particular, as estimates of fungal mass in roots and the impact of mycorrhizal inoculation programs have not been characterized using the proposed biochemical assays.

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CHAPTER 2

Ontological and environmental influences on ergosterol content and activities of polyamine biosynthesis enzymes in *Hebeloma crustuliniforme* mycelia[1]

2.1 Introduction

Measurements of fungal mass and metabolic activity of mycorrhizal root:soil systems are needed to provide information to interpret the effectiveness of mycosymbionts in acquiring nutrients and affecting plant performance. Fungal impact on soil nutrient cycling and rhizosphere ecology could also be examined via such quantity/activity relationships.

Ideal biochemical indices of fungal mass would be stable along environmental gradients and insensitive to ontogenic changes. Low background levels in solid substrates and a lack of interferences during analysis are also desired. Current biochemical techniques to quantify fungal mass include: 1) fluorescein diacetate (FDA) staining to distinguish viable hyphae from dead (Soderstrom 1977); 2) chitin assay to quantify fungal cell walls (Uchida and Yamaguchi 1984); and 3) quantification of ergosterol, a fungus-specific membrane component (West *et al.* 1987). The former two methods are difficult to interpret in soil and mycorrhizal root systems. Quantification of FDA-active soil hyphae is made difficult by the destruction of significant amounts of hyphae during sample preparation (Soderstrom 1979) and non-fungal amino sugars in soil may confound the chitin assay (Parsons 1981).

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1. A version of this chapter will be submitted to the Canadian Journal of Microbiology for publication. (Johnson, B.N. and W.B. McGill).

Ergosterol ($\Delta^{5,7,22}$ ergostatrienol) is present in the plasmamembrane where it facilitates condensation of phospholipid layers, thereby permitting proper orientation of fatty acyl chains and controlling membrane fluidity (Nes and McKean 1977). Sterols are also present in fungal mitochondria (Peltauf and Schatz 1969). Ergosterol is a membranous sterol of ectomycorrhizal fungi within the Ascomycotina and Basidiomycotina subphyla (Weete 1974) and is of only minor significance in most plant tissues (Nes 1977). By this exclusiveness, Seitz *et al.* (1979) suggested that ergosterol be monitored as an indicator of fungal mass where hyphae cannot be separated from a solid substrate or host tissue.

Ergosterol assays have been used to describe fungal mass in soil (West *et al.* 1987), cereal grains (Seitz *et al.* 1979) and mycorrhizal roots (Salmanowicz and Nylund 1988) however, such applications create the need to describe how ontogeny and environment influence the ergosterol content of mycelia. Huang *et al.* (1985) reported 2– to 4–fold increases in ergosterol of button stage mushrooms compared to stalks and ergosterol content varied with sporulation rate and degree of hypha vacuolation. Variations within colonies of mycorrhizal fungi have not been described. Further, Foster (1949) reported optimal glucose:urea ratios for maximum sterol content and concluded that media composition affects sterol synthesis. Presently, there are no descriptions of environmental influences on ergosterol content of mycorrhizal fungi. Such descriptions will be undertaken here.

Estimates of fungal mass in soil and mycorrhizal roots may be complemented by measurements of metabolic activity. Activity assays should be free of interferences from solid substrates and be subject to only minor abiotic contributions. The ideal activity estimate should also be an indicator of instantaneous condition, not biased by previous environmental conditions; the ideal enzyme for assay would be inducible and exhibit rapid and reversible responses to environment (Nannipieri *et al.* 1983).

Ornithine decarboxylase (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19) activities have rapid turnover times (less than 30 min; Pegg and Williams–Ashman 1981) and thus, cytosolic activity levels vary to reflect enhancement or inhibition by external stimuli at the time of sampling. Reaction products of ADC and ODC are polyamines (PAs), low molecular weight nitrogenous bases involved in cytosolic regulatory processes. Putrescine (PUT), generated directly from ODC and indirectly from ADC, is converted to spermine (SPM) and spermidine (SPD) (Walters *et al.* 1985). These polyamines affect electrostatic properties of membrane–bound enzymes responsible for nutrient uptake (Riedell 1987). Cytosolic SPD is essential in the formation of associable 30s ribosomal subunits (Agranati and Goldemberg 1977) while also reducing error frequency in translation events for protein synthesis (Jelenc and Kurland 1979). Nuclear SPD stimulates the rate of chromosomal replication; it serves as a cofactor to DNA gyrase which maintains DNA in an underwound tertiary configuration (Weimer *et al.* 1975). Spermidine affects tRNA conformation and crystalline structure by catalyzing the association of tRNA with amino acids (Loftfield *et al.* 1981). Exogenous PA applications enhance RNA synthesis (Cohen 1971) and transcription and translation rates (Stevens, 1970). As polycationic moieties, PAs bind to phospholipid heads and other anionic sites on membranes, thereby affecting membrane stability and function (Slocum *et al.* 1984). Typically, they decline with age and are highest in actively growing plant tissue (Roberts *et al.* 1986). Their apparent association with macromolecule synthesis has led to rates of PA production being suggested as analogues of growth rate (Suresh *et al.* 1978; Fuller *et al.* 1978; Walters *et al.* 1985).

Activities of PA biosynthetic enzymes may be used to monitor specific biotic activities in soil and root systems provided influences of ontogeny and environment on levels of activity are known. Such influences have not been reported for pure–cultured mycorrhizal fungi.

2.2 Objectives

The overall aim of this study was to determine if ergosterol concentration and activities of polyamine biosynthesis enzymes vary within vegetative mycelia of *Hebeloma crustuliniforme* (Bull.St.Amans) Quel, a basidiomycete ectomycorrhizal with ecomonically–important conifers on the eastern slopes of the Rocky Mountains of Alberta. Using pure batch cultures, the specific objectives were to:

1) determine if ergosterol concentration and activities of polyamine biosynthesis enzymes in mycelia vary with stage of maturity; and

2) determine if glucose–C and inorganic–P alter ergosterol concentration and activities of polyamine biosynthesis enzymes in actively growing mycelia.

2.3 Materials and Methods

2.3.1 Fungal isolate origin

An isolate of *H. crustuliniforme* was obtained from the University of Alberta Mycological Herbarium (UAMH isolate No. 5310). It was collected in September of 1985 from a *Pinus contorta/Picea glauca* stand near Grande Prairie, Alberta (Sec 10, Tp65, R2, W6; 54.3^{o}N lat.; 118.15^{o}W long.) and maintained in liquid nitrogen.

2.3.2 Experimental protocol

Variations in ergosterol content and enzyme activities within colonies.

Mycelia of *H. crustuliniforme* were cultured on Petri dishes in Melin–Norkrans medium with agar (20 g L^{-1}). Complete modified Melin Norkrans medium (Molina and Palmer 1982) contained (per L): 3 g malt extract, 10 g d–glucose, 0.25 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4, 0.15 g $MgSO_4$–$7H_2O$, 0.05 g $CaCl_2$, 0.23 g NaCl, 0.6 mg $FeCl_2$ and 0.1 mg thiamine–HCl. PH was adjusted to 5.6 with H_2SO_4 or NH_4OH. Solutions were autoclaved at 120 ^oC for 30 min and cooled to 60 ^oC before being plated.

Mycelia were introduced to 4 Petri dishes as 3 mm circular plugs sampled from the perimeter of one source colony. Plates were incubated at 24 ^oC. Colonies were separated from agar by sterile cellulose–acetate papers (0.45 um pore diameter; 5 cm paper diameter; Fisher Scientific LTD., Edmonton) which permitted removal of fungal hyphae for dry mass determination.

Mycelia of variable maturation states were obtained by sampling core, midcolony and perimeter colony positions with a 5 mm cork borer and scalpel. Four replicated colonies were used for measurements of ergosterol content and activities of ADC and ODC of the colony regions.

Temporal changes in whole colony ergosterol content and enzyme activities.

Erlenmeyer flasks (125 mL) with 75 mL Melin–Norkrans medium and agar (20 g L^{-1}) were used to cultivate *H. crustuliniforme* mycelia. Mycelia on cellulose-acetate papers were placed in flasks which were stoppered and incubated for up to 50 days at 24 ^oC. Four whole colonies were removed and colony dry mass, ergosterol content and enzyme activities determined at each sampling date.

Influences of medium composition on ergosterol content and enzyme activities.

Mycelia of *H. crustuliniforme* were cultured in four variations of Melin Norkrans medium. Medium variations were designated as '–C,+P', '+C,+P', '–C,–P' and '+C,–P' to indicate the presence or absence of glucose–C and/or PO_4–P. For media without PO_4–P enrichment, $(NH_4)_2HPO_4$ and KH_2PO_4 were substituted by 0.37 g KNO_3 and 0.02 g NH_4NO_3. At 24 days, ergosterol analyses and enzyme assays were performed on perimeter sections of 8 to 11 colonies.

2.3.3 Analytical Biochemistry

Ergosterol assay.

Ergosterol was assayed using an equilibrium extraction procedure (Seitz *et al.* 1979) which takes advantage of the negligible water solubility of sterols (Capek *et al.* 1966). Three mL of anhydrous methanol with 0.14 g KOH was added to samples in threaded 10 x 150 mm tubes. Tubes were heated at 100 ^{o}C for 5 min. Cooled alcoholic solutions were shaken with 2.5 mL petroleum ether for 2 min. Petroleum ether was pipetted off, deposited in glass vials and evaporated at 4 ^{o}C. Sterol residues were redissolved in 150 mL ethyl acetate and ergosterol was quantified by gas chromatography using a 'Gas Chrom Q' column with 'OV–17' (Mandel Scientific Ltd., Guelph, Canada). Injection volume was 6 to 9 uL with He as the carrier and testosterone as the internal standard. Injection, column and FID detector temperatures were 305, 250 and 285 ^{o}C, respectively. Four equilibrium extractions were performed. This technique recovered 97 $\pm$ 2.1% of ergosterol added to standards in preliminary trials.

Assay of ADC and ODC activities.

Enzyme activities were determined by incubation of enzyme extracts with ^{14}C–carboxyl–labelled substrates using a modification of the the method of Walters

et al. (1985). Fresh mycelia were ground at 5 ^{o}C in 400 *u*L of 25 mM Tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 7.6 for ADC; 8.0 for ODC) with 50 *u*M pyridoxal phosphate and 2.5 mM dithiothreitol and transferred to 1.5 mL centrifuge tubes with 3 x 200 *u*L aliquots of buffer. Following centrifugation (20,000 x G; 10 min), pellets were dried at 60 ^{o}C for 18 hr. Aliquots of the crude enzyme extract (400 *u*L) were transferred to 1.5 mL tubes and placed into 7 mL plastic vials containing 300 *u*L 0.5M NaOH. Fifteen *u*L of ^{14}C-labelled ornithine or arginine in buffer (3.08 kBq total activity) was added to each tube. Final arginine and ornithine concentrations (0.7 mM and 3.0 mM, respectively) were 20 to 30-fold above previously reported K_m values of 0.03 mM for arginine and 0.1 mM for ornithine (Applebaum *et al.* 1977; Pegg and Williams-Ashman 1981). Vials were fitted with rubber septa, wrapped with parafilm and placed in a shaker/incubator (37 ^{o}C; 40 oscillations min^{-1}). Enzyme reactions were stopped after 3 hours by injecting 150 *u*L 3% trichloroacetic acid. Radioactivity of NaOH solutions in Hionic-fluor (Packard Instrument Co., Donners Grove, Illinois) was determined using efficiency tracing (Ishikawa *et al.* 1984) with a Packard 2000A Liquid Scintillation Analyzer. Enzyme activities were expressed as nmols CO_2 evolved h^{-1} g^{-1} dry mycelia or nmols CO_2 h^{-1} mg^{-1} ergosterol.

2.3.4 Statistical Analyses

Significance of treatments on dependent variables was determined by analysis of variance using the linear additive model (Steel and Torrie 1980). Multiple means comparisons were performed by Fisher's LSD (Mize and Schultz 1985). Simple regression and multiple regression models were used to predict colony mass.

2.4 Results

2.4.1 Ontological influences on mycelia ergosterol content and enzyme activities

By 28 days, colonies were approximately 18 mm in radius. Colony 'core' constituted a 10 mm diameter zone of mycelia at the colony center. Mycelia 5 to 11 mm from the center constituted the 'midcolony' sample and mycelia 11 to 18 mm were classed as the 'perimeter' sample. Thus, core, midcolony and perimeter zones occupied 78, 300 and 640 mm^2 of the colony, respectively. Mycelia density decreased 11-fold from the colony core (3.20 ± 0.08 mg dry mycelia mm^{-2} surface) to the perimeter (0.27 ± 0.05). Density at the midcolony zone (0.67 mg mm^{-2}) was over 2 times the perimeter density.

Ergosterol concentration and enzyme activities were not uniformly distributed within the colony (Table 2.1). The core accounted for 40% of colony mass, yet contained less than 30% and 20% of the total colony ergosterol and polyamine biosynthesis potential, respectively. The perimeter, which contained less of the total colony mass than the core, contained 14% more ergosterol and 2.5 times greater polyamine biosynthesis potential than the core.

Mycelia ergosterol concentration, expressed on a dry mass basis, did not differ between the midcolony and perimeter although values for these locations were 45% greater than for the core (Table 2.2). Polyamine biosynthesis potential per unit dry mass increased toward the colony perimeter (Table 2.3). Arginine decarboxylase activity was 3.5 times greater and ODC activity 2.6 times greater at the perimeter. Core activity values were 28% and 39% of perimeter for ADC and ODC, respectively (Table 2.3). This yielded increasing ODC/ADC ratios with increasing proximity to the core.

Expressed on an ergosterol mass basis, ADC activity was 2 fold greater in the perimeter mycelia than in the core and midcolony zones which did not differ significantly (LSD; $p < 0.05$; Table 2.4). Ornithine decarboxylase activity per ug of ergosterol was slightly but not significantly higher at the perimeter than at the other two locations. The ratio of ODC to ADC activity was 2.5 times higher in the core than in the perimeter, with an intermediate value for midcolony mycelia. Ergosterol concentration was positively correlated with total mycelial activity of polyamine biosynthesis enzymes (Table 2.5).

2.4.2 Temporal changes in colony ergosterol content and enzyme activities

As the incubation proceeded to 50 days, colony dry mass increased by 2 orders of magnitude, from 7.9 to 736 mg per colony (Figure 2.1). Mean quantities of ergosterol per colony increased; the 50–day mean was 34 times the value at the start of the experiment (Figure 2.2). During the incubation, colony activities of ADC and ODC increased 30– and 89–fold, respectively (Figures 2.3 and 2.4).

Mean ergosterol concentration in mycelia calculated for each sampling date declined significantly over the course of incubation (Table 2.5). Values for the mycelia mass used for initital inoculation were 2.6 fold greater than in colonies at 50 days. Activities of ADC declined approximately 3 fold. Mass–based ODC activities were lower at early sample dates but did not vary with time.

A regression equation was expressed using colony mass as the dependent variable and ergosterol concentration as the independent variable. Ergosterol values of two samples collected at 40 days were rejected for residing outside the 95% prediction limits for the ergosterol content, given their colony masses. The equation was $Y = -5.46 + 0.38(X)$, where Y = mycelial mass (mg) and X = ergosterol detected (ug colony^{-1}). Coefficient of determination (R^2) was 0.77 ($p < 0.05$).

2.4.3 Media composition influences on ergosterol content and enzyme activities

Ergosterol concentration of mycelia grown in the absence of glucose–C was higher than in glucose–amended media (Figure 2.5). The media lacking inorganic PO_4^{3-} yielded mycelia with significantly lower ergosterol values than corresponding media containing P. Similarly, colony mass was reduced on the '–P' media. Reduction of colony mass by omission of glucose–C was not significant.

Comparing '+C' media, ODC and ADC activities were significantly greater without inorganic–P addition (Figure 2.6). Among '–C' media, ODC and ADC activities were greater in the absence of PO_4^{3-}–P, although the difference was not significant for ODC. Glucose influences on ADC and ODC activities depended on inorganic P status of the environment (Figure 2.6). Activities of both enzymes increased upon glucose addition in the absence of P. The effect was particularly pronounced for ODC, where '+C,–P' activity was nearly five–fold that of the '–C,–P' case. With added P, glucose addition did not alter ADC activity, whereas ODC activity was increased by glucose addition.

Ratios of ODC:ADC activity varied among treatments. Inclusion of glucose–C increased the ratio by 3 fold for the '–P' case and 2 fold for the '+P' case. Media P status had an insignificant influence on ratios of ODC:ADC activity.

2.5 Discussion

Ergosterol concentrations are within a range of values previously described for entire 14 to 28 day–old *H. crustuliniforme* colonies (Salmanowicz and Nylund 1988) and are within the range of values described for other mycelia of other basidiomycetes (Weete *et al.* 1985; Huang *et al.* 1985). Ontological influences on metabolic activity and ergosterol concentration are indicated by differences among colony locations. Griffin (1982) noted that spherical pellets of mycelia have

impeded O_2 and nutrient movement. Growth and branching of inner hyphae may also be physically restrained. Negative correlations of mycelia density with PA biosynthesis (Table 2.5) support this.

Trinci (1971) demonstrated that synthesis of new hyphal wall material and forward migration of the protoplast keeps colony perimeters growing faster. Colony location differences in ergosterol concentration (Table 2.2) and polyamine biosynthesis potential (Table 2.3) support the notion of localized increases of membranous and cytosolic materials at the colony perimeter; peripheral hyphal apices are the major metabolic contributors to colonies, and a large fraction of dry mycelial mass near the core will be composed of vacuolated hyphae (Deacon 1980; Toledo *et al.* 1986). The extent to which sterols are translocated during hypha vacuolation has not been described, nor are there reports of differential rates of sterol synthesis and degradation within colonies. Examination of such developmental processes are necessary to describe causes of the intracolony variations in ergosterol content in Tables 2.1 and 2.2. Sterols are obligate cofactors of proteinaceous carriers and integral enzymes (Elliot *et al.* 1977) and serve a role in maintaining viability of cell membranes and mitochondria and are more abundant in viable hyphae. Nevertheless, the regression of colony mass on ergosterol mass accounts for 77% of the variability in colony mass.

Activities of polyamine biosynthesis enzymes differ significantly among colony locations (Table 2.3), with proportions of total colony activity increasing toward the perimeter (Table 2.1). The perimeter is the dominant region of hyphal growth thus, activities of the enzymes may serve as analogues of metabolic activity. However, the use of ADC and ODC as markers of overall metabolic activity requires more substantive verification, likely in the form of establishing correlations between enzyme activities and other indices of activity, such as ATP content.

Seitz *et al.* (1979) stated that the usefulness of an assay as an indicator of fungal mass or activity may be determined by comparing initial amounts of the fungal component in a substrate to increases as fungi colonize the substrate. Total ODC activity and ergosterol abundance increased as colonies grew and paralleled increases in fungal mass during a 50–day incubation. Therefore, fungal dynamics may be described by monitoring changes in ergosterol content and ODC activity in axenically–cultured mycorrhizal roots or root–fungal pathogen systems.

An effect of environmental conditions on mycelia ergosterol content and polyamine biosynthesis potential is supported by Figures 2.5 and 2.6. Variations in ergosterol concentration are not thought to reflect ontological differences in this case, but rather morphological responses of hyphae to treatments. Fungal morphology was not examined here; further examination of fungi differing in morphological states may clarify such influences on ergosterol concentration. The '–P' media yielded significantly lower ergosterol concentrations than in respective cases where P was added and thus, fungal mass accumulation was impaired by P deficiency (Figure 2.5). Rodriquez and Parks (1983) observed a correlation of ergosterol concentration to growth rate in *Gibberella fujikuroi* cultures; results here are consistent with these data.

Activities of polyamine biosynthetic enzymes of *Hebeloma crustuliniforme* aerial mycelia were also sensitive to external nutritional conditions (Figure 2.6). ODC and ADC activities were stimulated by carbon supplements, regardless of P status and thus, the fungus exhibited a metabolic response to energy enrichment. Increased enzyme activities here may indicate increases in the rate of cell division; such a relationship has been demonstrated in plants (Galston 1983).

Arginine decarboxylase and ODC activities were greater without inorganic P addition to the medium. High levels of polyamines are associated with actively growing tissue (Schwartz *et al.* 1986) however, mycelial mass accumulation was not enhanced by deletion of inorganic P. Previous authors have demonstrated increased internal PUT concentration with potassium stress (Slocum *et al.* 1984) and low media pH (Young and Galston 1983). A similar event may be inferred here; elevation of enzyme activities in the P–deficient medium may have been associated with the increased need for polyamine products. Cells would then have an enhanced ability to maintain membrane integrity (Riedell 1987) and thus prevent cytosol leakage.

Ratios of ODC:ADC activity in *H. crustuliniforme* mycelia were greater than unity (Table 2.3, Figures 2.3 and 2.4). The activity ratio increased towards the colony core and as colonies aged (Tables 2.1 and 2.6). Such a bias in putrescine production pathways has been documented in mammalian tissues and some invertebrates lack ADC activity entirely (Pegg and Williams–Ashman 1981). Similar surveys of higher fungi have not been reported. Analysis of activity ratios in mycological systems may provide further insight to the metabolic condition of the fungus.

Assays of ADC and ODC as analogues of fungal metabolic condition may be useful in interpreting influences of environmental condition on fungal activity and viability. However, as other pathways for the metabolism of L–[1–^{14}C]ornithine to $^{14}CO_2$ may exist (Pegg and Williams–Ashman 1981), the determination of PUT, SPD and SPM concentrations in tissues may provide additional information. There is also merit in examining mechanisms by which PA metabolism responds to external stimuli such as in these experiments. Therefore, general application of the assays would be conditional upon examination of such variations in organisms of study.

2.6 Conclusions

Ergosterol concentration of *H. crustuliniforme* mycelia were affected by ontogeny; mycelia at the perimeter of a colony contained up to 1.8 times more ergosterol than those in the core. Ergosterol concentrations of colonies under normal conditions averaged 3.74 $\pm$ 0.46 *ug* ergosterol mg^{-1} dry mycelia, but ergosterol concentration increased 3 to 4 fold with omission of glucose from the growth medium. Although the ergosterol concentration of mycelia varied, a regression-based conversion may be used to estimate mycelia mass from ergosterol analyses. In complete Melin-Norkrans medium, 77% of the variation in mycelia mass was described by: $Y = -5.46 + 0.38(X)$; where Y = mycelia mass (mg) and X = ergosterol mass (*ug*). Application of similar regression-based ergosterol estimates of fungal mass may require that influences of nutritional environment be defined.

Activities of polyamine biosynthesis enzymes of *H. crustuliniforme* were also affected by mycelia ontogeny, with activities greater in actively-growing mycelia. Ornithine decarboxylase activity was 20 to 30 times greater than that of ODC in *H. crustuliniforme* in all colony locations and ADC activity decreased more upon maturation of mycelia. Thus, ratios of ADC:ODC activity may be useful indicators of mycelial activity in such pure systems.

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Table 2.1. Distribution of mass, ergosterol and ADC and ODC enzyme activites within H. crustuliniforme colonies.

Colony location	% of total area	% of total mycelia mass	% of total ergosterol mass	% of total enzyme activity ADC	% of total enzyme activity ODC
core	7.6 *a* *	40.4*b*	28.2*a*	14.8 *a*	19.0 *a*
midcolony	29.5 *b*	32.2*ab*	39.1 *b*	32.7 *b*	33.5 *b*
perimeter	62.9 *c*	27.3*a*	32.7*ab*	52.5 *c*	47.5 *c*

* within columns, means not followed by the same letters are significantly different, (LSD; $P < 0.05$).

Table 2.2. Ergosterol concentration of H. crustuliniforme mycelia sampled from core, midcolony and perimeter colony locations.

Colony location	mg ergosterol / g dry mycelia	
	mean	SEM*
core	3.19 *a***	0.30
midcolony	5.68 *b*	0.65
perimeter	5.63 *b*	0.81

* Standard errors of means are indicated (n=4).
** Means not followed by the same letter are significantly different (LSD; < 0.05).

Table 2.3. ADC and ODC activities per g of H. crustuliniforme mycelia sampled from core, midcolony and perimeter colony locations.

Colony location	Enzyme activity (nmol CO_2 evolved / g mycelia per hr) ADC mean	ADC SEM**	ODC mean	ODC SEM*	ODC/ADC*
core	4.62 *a****	(0.56)	137.7 *a*	(14.6)	29.9
midcolony	10.38 *b*	(1.09)	242.0 *a*	(24.1)	23.3
perimeter	16.54 *c*	(1.23)	353.0 *b*	(50.2)	21.4

* ODC/ADC indicates ratio of activities.
** Standard errors of means are indicated (n=4).
*** Means not followed by the same letter are significantly different (LSD; $P < 0.05$).

Table 2.4. ADC and ODC activities per mg ergosterol in H. crustuliniforme mycelia sampled from core, midcolony and perimeter colony locations.

Colony location	Enzyme activity (nmol CO_2 evolved / mg ergosterol per hr) ADC mean	ADC SEM*	ODC mean	ODC SEM**	ODC/ADC*
core	1.12 *a***	(0.08)	57.24 *a*	(7.06)	52.0
midcolony	1.58 *a*	(0.08)	55.34 *a*	(11.55)	36.9
perimeter	3.20 *b*	(0.41)	64.28 *a*	(4.72)	20.7

* ODC/ADC indicates ratio of activities.
** Standard errors of means are indicated (n=4).
*** Means not followed by the same letter are significantly different (LSD; $P < 0.05$).

Table 2.5. Matrix of correlation coefficients (r) for total enzyme activity, ergosterol concentration and mycelia density.

	mycelia ergosterol content (ug / mg mycelia)	mycelia density (mg mycelia / mm2 colony)
total enzyme activity (ADC + ODC) pmols CO2 evolved / mg mycelia	0.77 (.003)*	-0.73 (.007)
mycelia ergosterol content		-0.74 (.006)

* levels of significance based on Student's t distribution.

Table 2.6. Ergosterol concentrations and ADC and ODC activities of H. crustuliniforme mycelia during a 50 day incubation.

Incubation period (days)	Ergosterol*		Enzyme activities** ADC		ODC	
	Mean	SEM***	Mean	SEM	Mean	SEM
0	5.8	0.3	22.6	1.9	356	57
0.5	5.1	0.7	11.2	0.3	229	53
1	6.8	1.0	14.7	2.5	261	63
2	2.4	0.6	13.7	1.2	240	38
4	3.8	0.6	15.5	0.9	305	18
7	3.1	0.6	12.2	0.7	350	51
11	3.5	0.3	10.6	2.0	375	34
16	4.9	0.5	8.8	7.8	457	28
23	3.3	0.2	4.4	4.1	386	37
31	2.5	0.2	5.4	1.2	438	18
40	1.5	0.5	4.0	1.6	474	27
50	2.2	0.2	7.3	2.5	341	29

* mg ergosterol / g dry mycelia

** nmols CO_2 evolved / g dry mycelia per hr

*** standard errors of means are indicated.

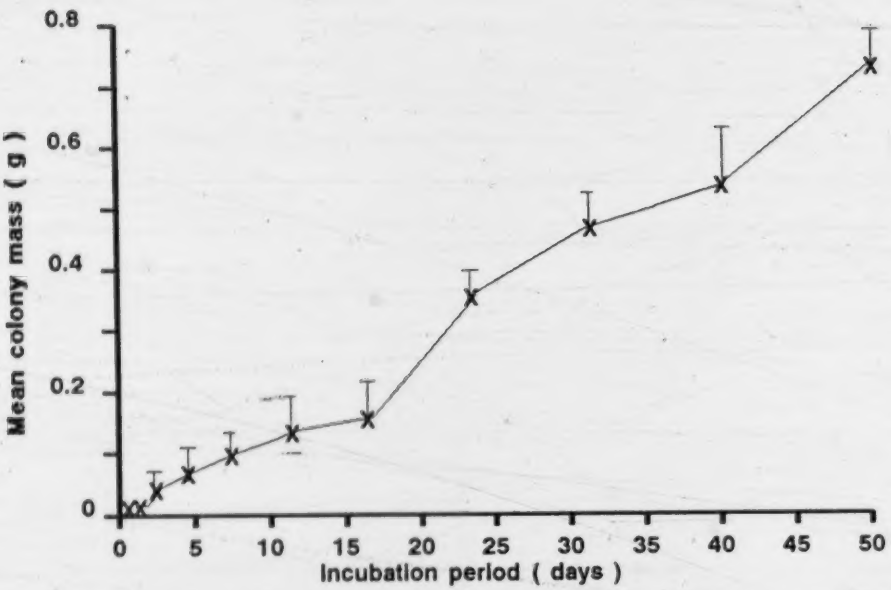

Figure 2.1. Mean mass of H. crustuliniforme colonies sampled during a 50 day incubation. Bars indicate standard errors of means.

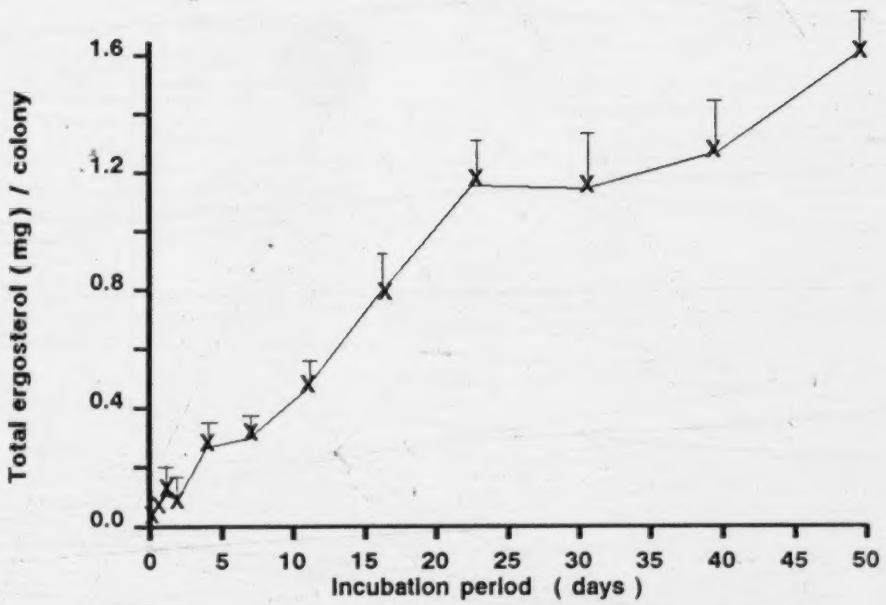

Figure 2.2. Total quantity of ergosterol in H. crustuliniforme colonies sampled during a 50 day incubation. Bars indicate standard errors of means.

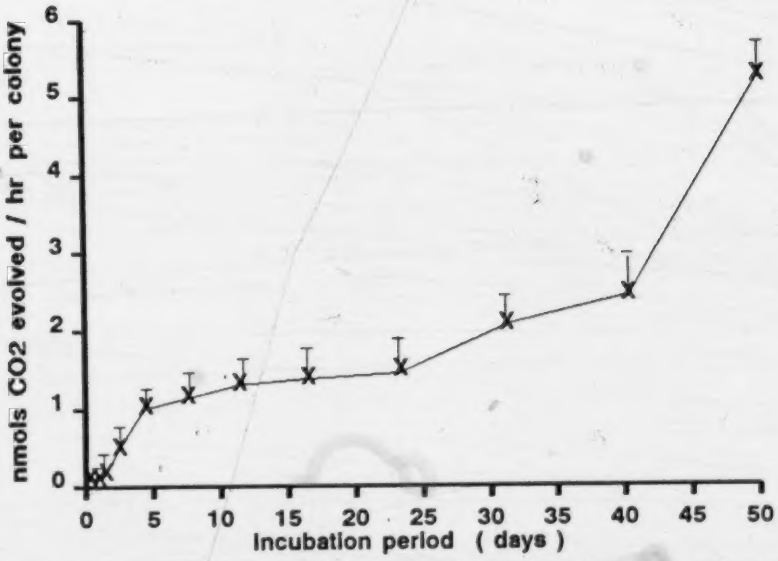

Figure 2.3. Total ADC activity of H. crustuliniforme colonies sampled during a 50 day incubation. Bars indicate standard errors of means.

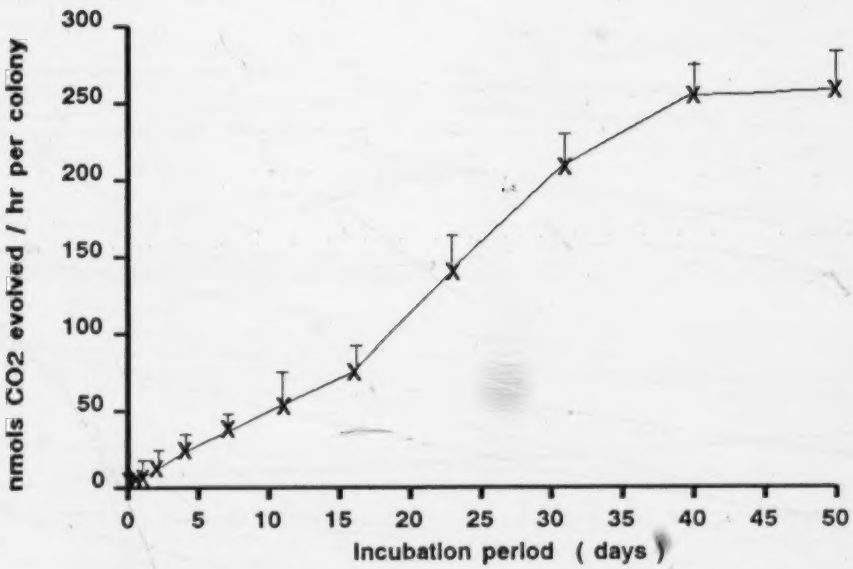

Figure 2.4. Total ODC activity of H. crustuliniforme colonies sampled during a 50 day incubation. Bars indicate standard errors of means.

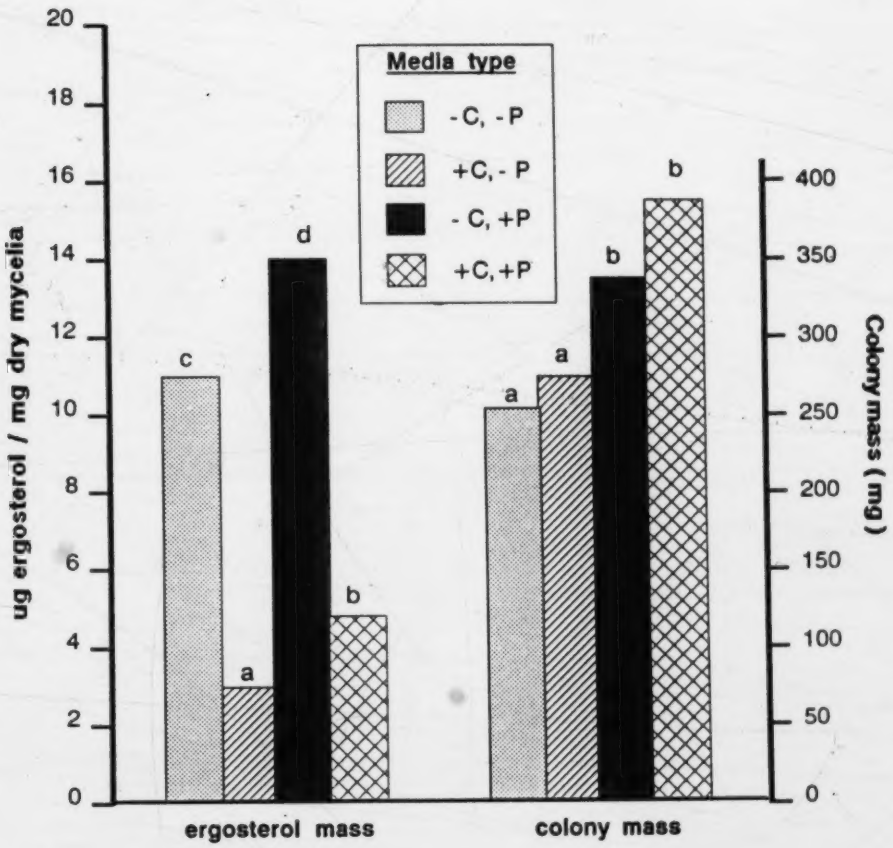

Figure 2.5. Ergosterol concentrations and dry mass of perimeter mycelia sampled from 24 day-old H. crustuliniforme colonies grown with or without added glucose-C or inorganic-P. Treatment means along the x axis contained 8, 11, 9 and 10 replicates, respectively. Means not followed by the same letter are significantly different (LSD; $P < 0.05$).

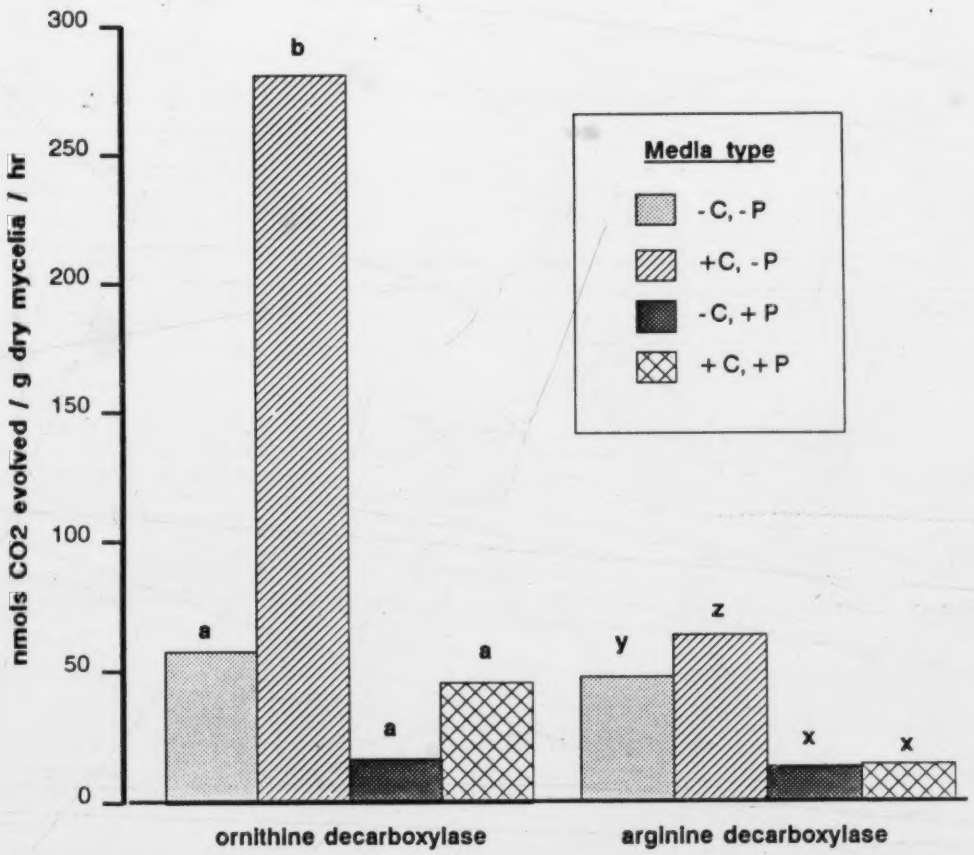

Figure 2.6. Arginine decarboxylase and ornithine decarboxylase activities of of perimeter mycelia sampled from 24 day-old H. crustuliniforme colonies grown with or without added glucose-C or inorganic-P. For each enzyme, means not followed by the same letter are significantly different (LSD; $P < 0.05$).

CHAPTER 3

Stability and origin of ergosterol and activities of polyamine biosynthesis enzyme activities in a loamy sand[2]

3.1 Introduction

There often is a need to determine the mass of various taxa in some studies of soil ecology because residue degradation, nutrient immobilization, plant diseases and root symbiotic associations are among many processes influenced by soil community structure. Bacterial and fungal populations are commonly enumerated as they are predominant biotic groups (Nakas and Klein 1980). One approach to quantify soil microflora is the fumigation–incubation procedure (Jenkinson and Powlson 1976) based on N and C mineralized from killed microbial biomass. The ratio of CO_2 evolved to N mineralized was strongly correlated to the ratio of fungal–to–bacterial biomass present prior to fumigation (Ingham and Horton 1987).

Biochemical techniques have been used to quantify fungal mass. Among them are fluorescein diacetate staining which allows living and nonliving hyphae to be discriminated (Soderstrom 1979) and analysis of ergosterol ($\Delta^{5,7,22}$ ergostatrienol), a fungal cell membrane component. The latter technique has recently been applied as a specific biochemical indicator of fungal mass in cereal grains (Seitz *et al.* 1979), soil (West *et al.* 1987) and mycorrhizal roots (Salmanowicz and Nylund 1988).

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2. A version of this manuscript will be submitted to Soil Biology and Biochemistry for publication. (Johnson, B.N. and W.B. McGill).

Ergosterol is a plasmamembrane component of many fungi including most basidiomycetes (Huang *et al.* 1985) and thus, monitoring its fate may indicate the rate at which fungi grow or decay in soil. However, such usage requires that the residence time of ergosterol be determined. Ergosterol may be retained in soils within viable hyphal membranes or a portion may be abiotic, stabilized onto colloidal material and resistant to microbial transformations. Chalal *et al.* (1966) reported that plant lipids were resistant to decomposition in peat. This was consistent with the suggestion by Whitehead (1963) that long chain fatty acids, which are poorly soluble in water and strongly adsorb to clay, may persist for long periods in soil. As a result, many lake sediments and petroleum and coal deposits contain aromatic organic entities of discrete biological origin (Mermoud *et al.* 1984, Cooper *et al.* 1986). Neutral lipids, predominantly C-29 and C-27 sterols from plant and microfaunal sources respectively, are often extracted.

The nature and extent of transformations of neutral lipids depends on the amount and type of material and the depositional environment. In mineral material, sterols are subject to conjugation, cleavage, hydroxylation, reduction, oxidation, dealkylation, dehydrogenation and aromatization reactions (Capek *et al.* 1966, Cooper *et al.* 1986). Ergosterol stability in soil has not been described and therefore, its utility as an index of fungal mass is uncertain.

Soil microbial activity may be examined by enzymological methods (Skujins 1978; Reddy *et al.* 1987). However, such assays commonly include an abiotic factor contributed by extracellular enzymes adhering to inorganic colloids or dead cell fragments, and active enzymes within dead cells or associated with living microbial cells (Ladd 1978). Arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), enzymes integral to the formation of polyamines, have not been applied to studies of soil biota, although they have been used in growth studies of pure-cultured bacteria and mammalian tissue (Pegg and Williams-Ashman 1981).

For several reasons, ADC and ODC activities of polyamine (PA) biosynthetic enzymes are good candidates to improve understanding of soil microbial activity. First, the enzymes have been strongly correlated to the growth rate of many biological systems (Galston 1983; Schwartz *et al.* 1986). Second, the enzymes are ubiquitous constituents of growing bacterial, algal, fungal and faunal cells, thus ensuring diverse soil microorganisms to be included in the assay (Pegg and Williams–Ashman 1981). Third, the enzymes are highly inducible and have a short half–life (Slocum *et al.* 1984); polyamine biosynthetic activities of tissues reflect metabolic status at the sampling time and are less likely to be biased by past organism responses to environmental conditions. Despite possible advantages, activities of ADC and ODC have not been included in studies of soil microbial activity, nor has any attempt been made to use such assays in conjunction with studies of soil microbial biomass.

3.2 Objectives

The aim of this study was to examine the suitability of analyses of ergosterol, ADC and ODC to studies of fungal mass and biotic activity in soil. Using mineral soil material incubated with or without fungi or ergosterol, the specific objectives were to:

1) determine the extent to which ergosterol is stabilized or metabolized in a loamy sand by examining its fate when added in free form or bound within cell membranes.

2) determine the extent to which ADC and ODC activities exhibit temporal variations in soil and the extent to which they are affected by the addition of fungal mycelia.

3.3 Materials and Methods

3.3.1 Fungal isolate origin and cultivation

Vegetative mycelia of the *H. crustuliniforme* isolate described in Chapter 2 were labelled with ^{14}C by cultivation on ^{14}C–glucose–enriched Melin Norkrans medium (Molina and Palmer 1982). For mycelia added directly to soil, the specific activity was 1.43 kBq mg^{-1} glucose–C; for mycelia processed for ^{14}C–ergosterol, the specific activity was 1.004 kBq mg^{-1} glucose–C. Mycelia were grown on cellulose acetate papers in 125–mL Erlenmeyer flasks placed in 2 L Mason jars and incubated with 1.0M NaOH. Growth was in the dark at 24 ^{o}C for 20 days with NaOH solution replaced at 10 days.

3.3.2 Soil origin and characteristics

Soil was obtained from the 0–15 cm depth of a natural lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* englem.) stand developed on aeolian parent material west of Rocky Mountain House, Alberta (Sec 10, Tp38,R8; 52.15^{o}N lat.; 115.0^{o}W long.). The Bm horizon from a Prentice Soil Series was selected becuase it had a low pH (5.0 in water; McLean 1982), low inorganic P (2.3 mg kg^{-1} as Bray–extractable P; Olson and Sommers 1982) and coarse texture (loamy sand by hand texturing; Alberta Agriculture 1988). The soil contained 3.6 g total C kg^{-1} (by dry combustion; Nelson and Sommers 1982) and 60 mg mineral N kg^{-1} (by KCl extraction; Keeney and Nelson 1982). Total cation exchange capacity was 5.0 cmol charge kg^{-1} soil (by ammonium acetate extraction; Thomas 1982). Air–dried soil was ground to pass through a 2 mm sieve and moistened with Melin–Norkrans solution without glucose (200 mL solution kg^{-1} soil) and 8.00 g subsamples deposited into 50 mL beakers.

3.3.3. Amendment of soil with ^{14}C labelled ergosterol or mycelia

Radioactive *H. crustuliniforme* colonies (specific activity = 0.42 kBq mg^{-1} dry mycelia) were processed for the separation of neutral sterols according to the ergosterol procedure (Chapter 2). Sterol residues were redissolved in benzene:acetone (98:2; v/v) and spotted onto silica gel coated TLC plates. Chromatograms were developed in benzene:acetonitrile (90:10; v/v) and ergosterol retained as the 0.35 R_f band. Ergosterol (specific activity = 0.86 Bq ug^{-1} ergosterol) was removed from the gel by redissolution in absolute ethyl acetate. Aliquots of the ergosterol solution were added to moist soils in incubation jars and soils stirred for 10 min. to allow ethyl acetate to evaporate. Enrichment was 5.0 *ug* ergosterol g^{-1} soil. Specific activity of soil was 4.3 Bq g^{-1} dry soil. Soil was incubated in the dark at 24 ^{o}C with beakers containing 10 mL of 0.5M NaOH.

Whole *H. crustuliniforme* colonies were homogenized for 30 sec. in a Waring blender in Melin Norkrans solution without glucose. Aliquots (1.7 mL) were incorporated into 8.0 g soil samples in beakers. Specific activity of mycelia was 0.601 kBq mg^{-1} dry mass. Soil samples received 30.47 mg mycelia (12.82 mg mycelial C ; 77.39 *ug* mycelial ergosterol) $beaker^{-1}$. Specific activity of soil was 2.2884 kBq g^{-1}. Soils were incubated in the dark at 24 ^{o}C with 20 mL of 0.5 M NaOH in each jar.

This experiment consisted of 3 inoculation treatments (nonamended control; ^{14}C-labelled ergosterol added; ^{14}C-labelled mycelia added) and 8 sampling dates (up to 42 days) with three replications of each treatment x date combination. At each sampling time, samples were subdivided for fumigation-incubation determination of biomass, enzyme activities and ergosterol content.

3.3.4 Analytical methods

$^{14}CO_2$ Measurements.

Radioactivity of 5 mL of NaOH solutions was determined in 15 mL vials using scintillant and instumentation described in Chapter 2. CO_2–C trapped in the remaining 5 mL of NaOH was determined by titration with standardized 0.05 M HCl.

Ergosterol assay.

The procedure described in Chapter 2 was used, with a maximum of 3 g of moist soil added to a test tube containing 6.0 mL anhydrous methanol with 0.28 g KOH. Results were expressed as *u*g ergosterol g^{-1} dry soil.

Assay of ADC and ODC activities.

The procedure described in Chapter 2 was used. A maximum of 3 g moist soil was ground at 5 ^{o}C in 2.6 mL of 25 mM TRIS buffer (pH 7.6, ADC; pH 8.0, ODC) containing 50 *u*M pyridoxal phosphate and 2.5 mM dithiothreitol and transferred to pre–weighed 50 mL centrifuge tubes with 3 x 800 *u*L washings with buffer. Aliquots (400 *u*L) of the crude enzyme extract were analyzed and enzyme activities expressed as pmols hr^{-1} g^{-1} dry soil.

Soil microbial biomass by fumigation–incubation.

Soil samples (4.5 to 5.0 g) were fumigated with purified chloroform for 18 hr. The fumigant was evacuated and soils incubated for 10 days at 24 ^{o}C. Non–fumigated soil was not used as a control in recognition of different microbial conditions that occur in fumigated and non–fumigated soils (Voroney and Paul 1984). Soil biomass–C was calculated from the C–mineralized using a conversion factor of 0.41 (Ingham and Horton 1987).

3.4 Results

In preliminary trials, recovery of ergosterol added to moist soil (75% field capacity) was 97% ± 2.1%. Freeze drying of ergosterol–amended soil decreased recovery by 45%. Thus, ergosterol analyses were performed on moist soils. The base level of ergosterol in nonamended soil was 2.4 ug ergosterol g^{-1} dry soil. Of the 5.0 *ug* free ergosterol added g^{-1} soil, 96% was recovered at time 0. Of the 9.7 *ug* mycelia ergosterol added g^{-1} soil, 99% was recovered at time 0.

Soil ergosterol concentrations at the start of the experiment were greatest in amended soils (Figure 3.1). Ergosterol concentration of mycelia–amended soil nearly doubled between 0 and 4 days, while an increase of 71% was observed in the nonamended soil. In the ergosterol–amended soil, ergosterol content did not exhibit an early increase, rather declined 7% by 42 days. Following initial increases within 4 days, ergosterol concentrations in the mycelia– and nonamended soils declined to 65 and 34% of maximum values, respectively, by 22 days.

Mycelia–amended soil contained highest biomass–C levels throughout the incubation, with a peak at 18 hr representing a 2.3 fold increase from time zero (Figure 3.2). At 22 days, the biomass–C levels were one–half of the starting value. Biomass–C in the control soil increased by 40% between 0 and 37 hrs and this was followed by a constant decline to negligible values. Biomass–C in the ergosterol–amemded soil increased 20% within 37 hours and remained stable at values greater than those of non–amended soil. Cumulative CO_2 evolution during the incubation period was nearly equal for all treatments (Table 3.1).

Ergosterol–C and mycelia–C differed in the extents to which ^{14}C was mineralized (Figure 3.3). By 42 days, 7% of ergosterol–^{14}C was mineralized, compared to a 53.5% loss of mycelia–^{14}C by 22 days. Rates of $^{14}CO_2$ release from mycelia were greater within 13 days of the start of incubation than from 13 to 22 days. Ergosterol–$^{14}CO_2$ evolution was slight after 7 days.

Specific activities of evolved CO_2 (Figure 3.4), soil ergosterol (Figure 3.5) and biomass (Figure 3.6) decreased over time in mycelia–amended soils. Rates of decline were greatest between 18 and 39 hrs. Specific activity of ergosterol decreased by 80% during the incubation (Figure 3.5) and that of biomass–C decreased by 53% from 0 to 18 hr (Figure 3.6).

Specific activities of CO_2 evolved in ergosterol–amended soils were less than those of mycelia–amended soils at all times and exhibited different trends (Figure 3.4). From 4 to 22 days a 3 fold increase was observed. Specific activity of ergosterol in the ergosterol–amended soil decreased and after 5 days, was 2 to 5 times higher than the value for the mycelia–amended soil (Figure 3.5). Specific activity of biomass–C in the ergosterol–amended soil increased over 13 fold between 0 and 42 days (Figure 3.6). Values for the ergosterol–amended soil converged with the mycelia–amended soil values as the incubation progressed.

In preliminary trials, dry soil had negligible ADC and ODC activities. Upon wetting and storage for 18 hr, ADC and ODC activities were 0.34 and 3.25 pmols CO_2 evolved hr^{-1} g^{-1} soil, respectively. Mycelia amendment added ADC and ODC activities of 0.03 and 1.43 pmols CO_2 hr^{-1} g^{-1}, respectively. Of this, 102% and 111% of the activities were recovered at time 0. Activities of both enzyme increased from 0 to 7 days in all treatments (Figures 3.7 and 3.8). Arginine decarboxylase activity was lower than that of ODC by an order of magnitude and increased a maximum of 65%. Ornithine decarboxylase activities increased up to 2.5 fold from 0 to 4 days in the control soil and dropped to remain lowest of all treatments from 7 to 22 days. At all times, ODC activity of mycelia–amended soil was greater than for the other treatments but from 13 to 22 days, this soil had lowest ADC activity.

3.5 Discussion

Pure ergosterol was stabilized to a greater degree than was ergosterol added within mycelia (Figure 3.1). This confirms the resistance of free neutral lipids to microbial attack described by Chalal *et al.* (1966), although lipophilic interactions with colloids may have contributed the low ergosterol recovery from dry soil. Turffit (1948) demonstrated that soil microbes may degrade cholesterol and use it as a sole C source. Such a process may be present here, as incorporation of free ergosterol into biomass yields increasing specific activity of the compound over time (Figure 3.6). The utilization of free ergosterol is not extensive however, as only 8% of ^{14}C–labelled ergosterol was respired by 42 days (Figure 3.3).

The absence of an ergosterol peak (Figure 3.1) and the presence of only a slight biomass peak (Figure 3.2) in the ergosterol-amended soil may reflect the biocidal effect of ethyl acetate used as a carrier for ergosterol. Biomass–C values in this soil are sustained beyond the period when the control soil contained nearly no biomass–C. This feature, and the gradual increase in ^{14}C– ergosterol respired, indicate ergosterol is a C and/or energy source for microbial populations.

Melin Morkrans medium may have affected CO_2 evolution. Control soil contained 3.6 mg total C g^{-1} soil in addition to 0.43 mg C added as malt extract. Release of CO_2 by 22 days represented 55% of C present. Cumulative respiration in the mycelia amended soil was 0.4 mg C g^{-1} soil above the control; this represented 25 % of mycelia C. A value of 41 to 49% was expected from work of Marumoto *et al.* (1984) and Voroney and Paul (1980). As cumulative CO_2 evolution from the control soil appears to be too high, effects of mycelia and ergosterol amendment on rates of respiration have not been resolved from the data.

The evolution of up to 50% of added ^{14}C as CO_2 within 22 days (Figure 3.3) is within the range described by Marumoto *et al.* (1982) for soils amended with ^{14}C–labelled microorganisms. The respiratory loss of ergosterol added in mycelia (Figure 3.1) and the decrease in biomass–C after an initial flush (Figure 3.2) suggests that fungi were being metabolized after the initial flush of activity within 4 days consumed labile substrates added with the fungus and malt extract in the Melin Norkrans medium. The reduction in specific activity of the ergosterol in the mycelia–amended soil (Figure 3.5) demonstrates synthesis of non–labelled ergosterol during fungal growth (Figure 3.1), which diluted the ^{14}C label in this fraction. Decomposition of ergosterol added as mycelia occurred concurrently with synthesis.

West *et al.* (1987) concluded that ergosterol analyses in soil represent live fungi and that the assay has utility in monitoring changes in fungal populations. Temporal changes in ergosterol content of soils in this experiment support this conclusion. Robertson *et al.* (1988) reported a constant total hyphal length during a 12 week incubation, while fluorescien diacetate–active hyphae decreased; filamentous hyphae had a long residence time. Nelson *et al.* (1979) reported fast turnover times (1.6/week) for cell wall materials of filamentous fungi but, results here suggest that decomposition of mycelial mass may not be so rapid.

Ergosterol content of the non–amended soil (Figure 3.1) was stable after an initial increase. The uniform level of ergosterol is presumed to reflect constant mycelial mass (Chapter 2). Ergosterol isolated from mycelia and then added to soil appears in the biomass (Figure 3.4) and is respired (Figure 3.3) but, restrictions on its entry into biomass for assimilation or respiration were apparent. Biomass–C in the ergosterol–amended soil peaked within 4 days, while the amount of ^{14}C–ergosterol incorporated into biomass increased gradually over time. The early incorporation of ergosterol into biomass represented the assimilation of the exogenous sterol by soil microbes.

Membrane–bound ergosterol appeared to be lost from the soil biomass at a faster rate than free ergosterol is incorporated into biomass (Figure 3.9). There may be no natural circumstances where such quantities are applied to soil but indications here are that sterols will be gradually integrated into soil biota. There is a need, however, to analyze dynamics of ergosterol in other soils, with particular attention paid to influences of clay content and organic matter content on stability.

Changes in activity of ADC and ODC were evident during the incubation however, the biological significance of such activities is not clear. Melin Norkrans medium induced activity flushes similar to those of amended soils. Arginine decarboxylase activity remained high at 22 days in the ergosterol–amended soil although the biomass–C and CO_2 respiration had declined. At all sampling times, biomass–C in the mycelia–amended soil was 3 orders of magnitude greater than other soil treatments (Figure 3.2) yet, enzyme activities were nearly equal among soils (Figures 3.7 and 3.8). Thus, while ergosterol is an indicator of fungal mass, enzyme data appear to reflect activity of populations of various soil taxa.

3.6 Conclusions

It is possible to recover ergosterol from a loamy sand. Cell–free ergosterol and membrane–bound ergosterol were detectable but have different fates upon addition to soil. The ergosterol within viable tissues is metabolized and diluted into soil biomass, while free ergosterol is incorporated into biomass more slowly. Analysis of soils for ergosterol is useful as an estimate of fungal mass, as contributions from abiotic sources would be small. It is possible to detect activities of ADC and ODC in loamy sand with minimal interferences. Changes in activities of these enzymes during an incubation period are discernable. Activities of these enzymes are contributed by soil biotic groups of indeterminant origin.

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Table 3.1. Cumulative CO_2 evolved from loamy sand following addition of ergosterol or H. crustuliniforme mycelia. (mg CO_2-C evolved / g soil).

Soil Treatment	Incubation period (days) 0.75	1.6	3.6	6.6	10.6	15.7	21.7	41.7
control	0.3	0.5	1.0	1.3	1.6	1.8	2.2	-
mycelia added	0.2	0.3	1.3	1.7	2.3	2.6	2.6	-
ergosterol added	0.3	0.5	0.8	1.2	1.5	-	1.7	2.0

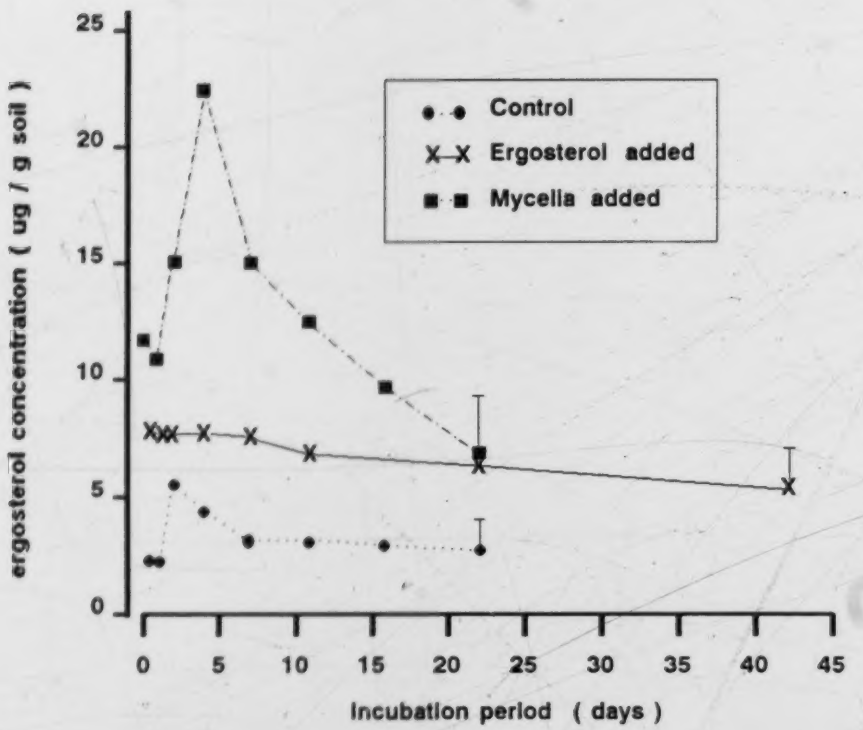

Figure 3.1. Ergosterol concentration of loamy sand incubated with or without added ergosterol or H. crustuliniforme mycelia. Bars indicate standard errors of means.

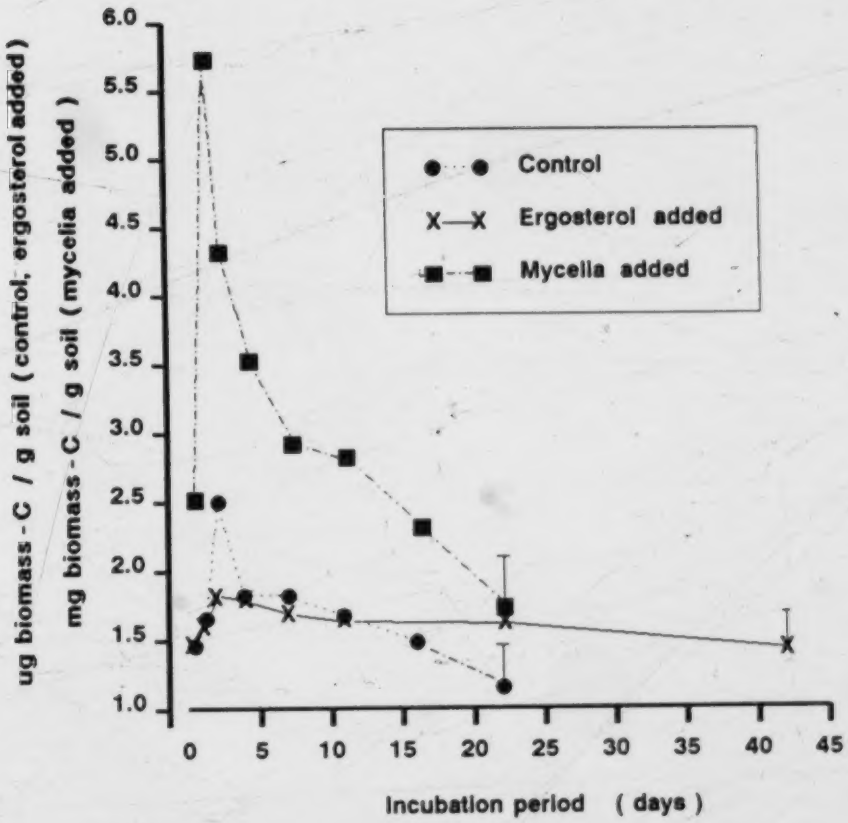

Figure 3.2. Biomass-C of loamy sand incubated with or without added ergosterol or H. crustuliniforme mycelia. Bars indicate standard errors of means.

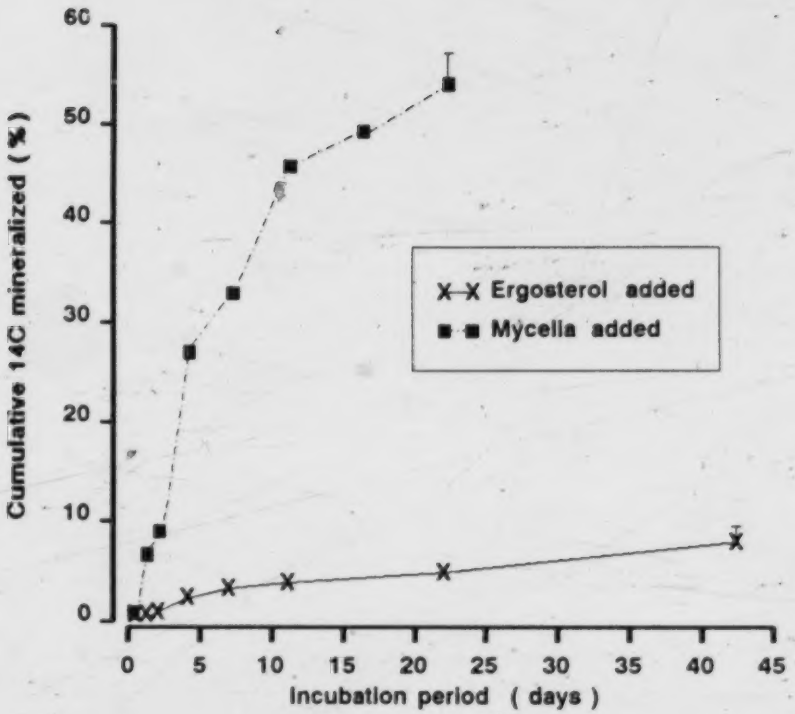

Figure 3.3. Cumulative evolution from a loamy sand of 14C added as ergosterol or H. crustuliniforme mycelia. Bars indicate standard errors of means.

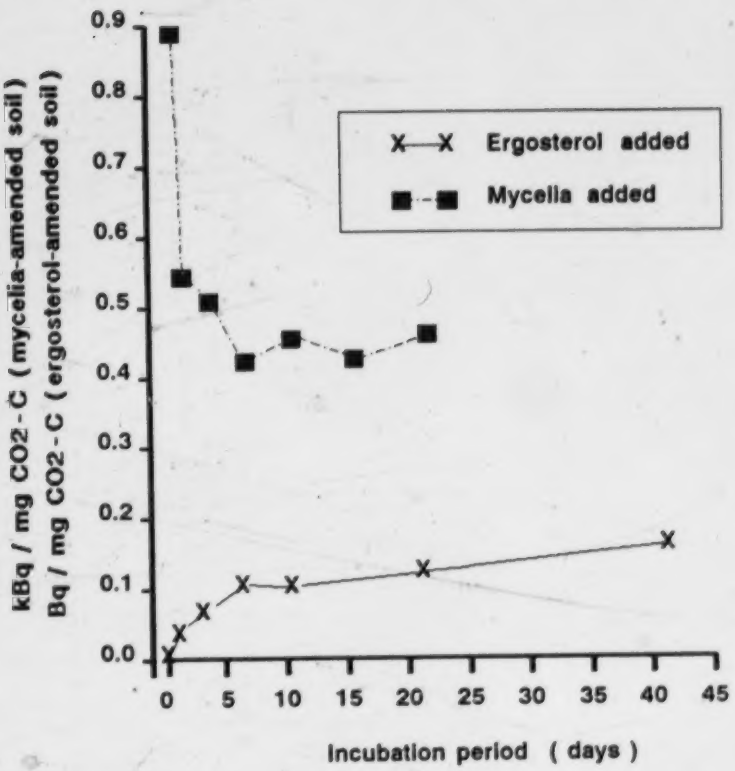

Figure 3.4 Specific activity of CO2 evolved from loamy sand with added ergosterol or H. crustuliniforme mycelia.

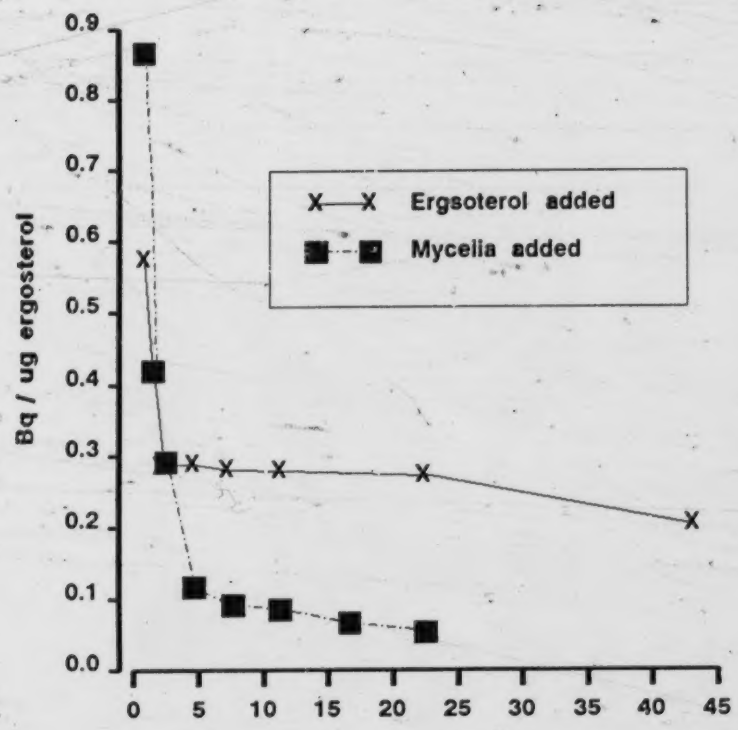

Figure 3.5 Specific activity of ergosterol in loamy sand incubated with added ergosterol or H. crustuliniforme mycelia.

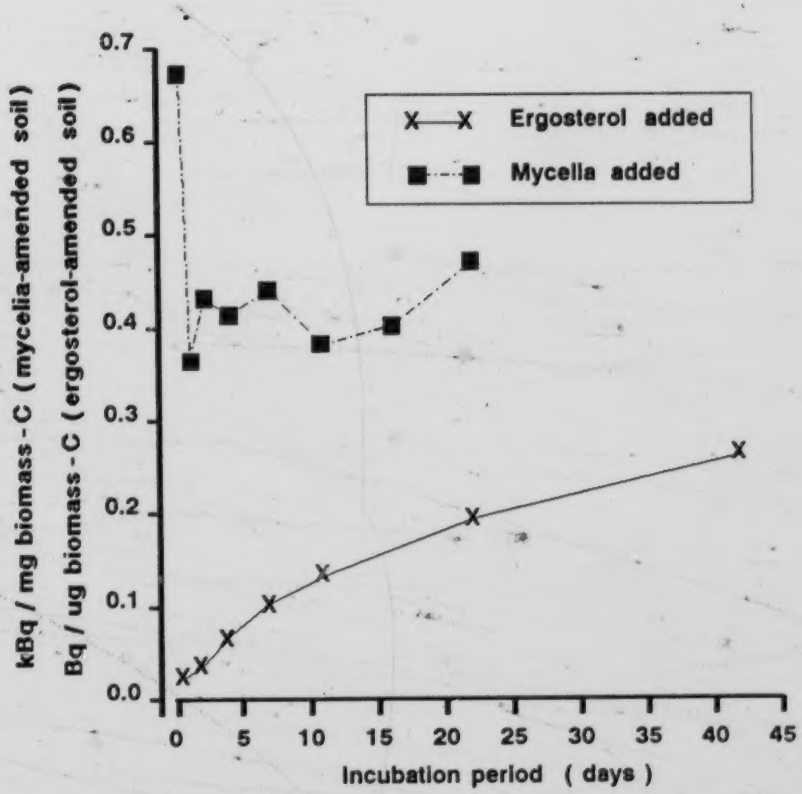

Figure 3.6. Specific activity of biomass-C in loamy sand incubated with ergosterol or H. crustuliniforme mycelia.

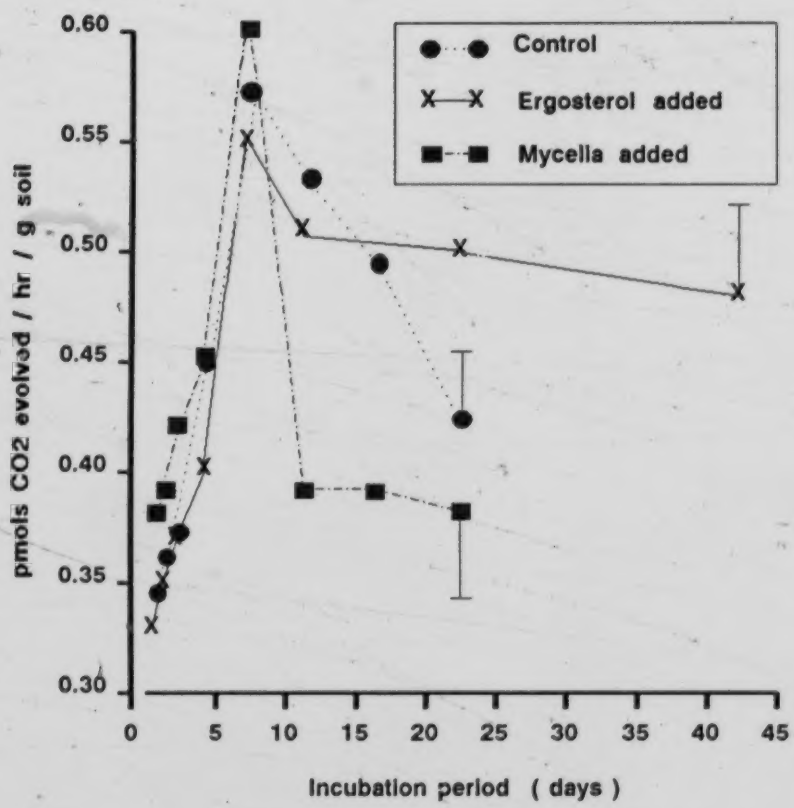

Figure 3.7. ADC activity of loamy sand incubated with or without added ergosterol or H. crustuliniforme mycelia. Bars indicate standard errors of means.

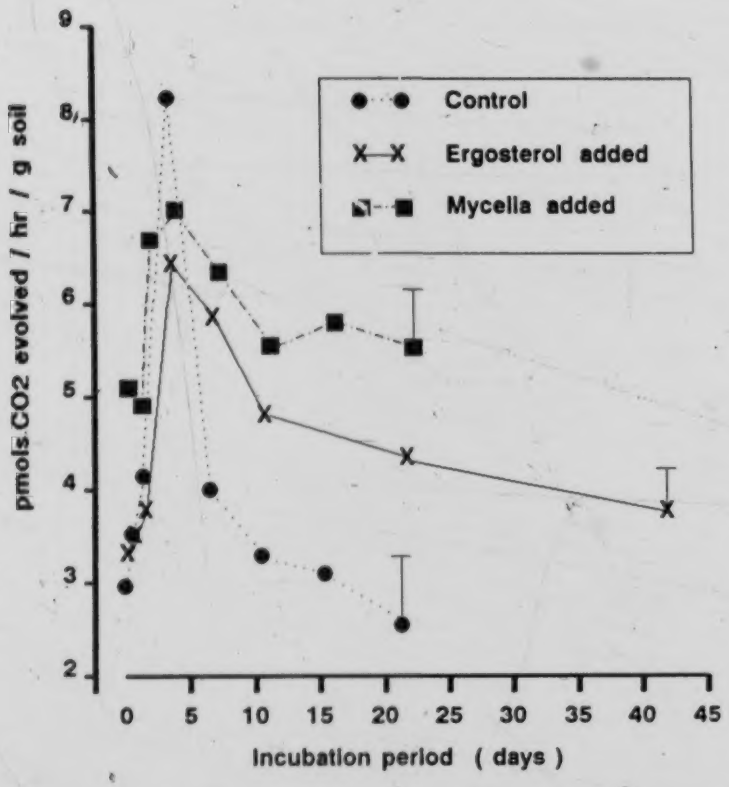

Figure 3.8. ODC activity of loamy sand incubated with or without added ergosterol or H. crustuliniforme mycelia. Bars indicate standard errors of means.

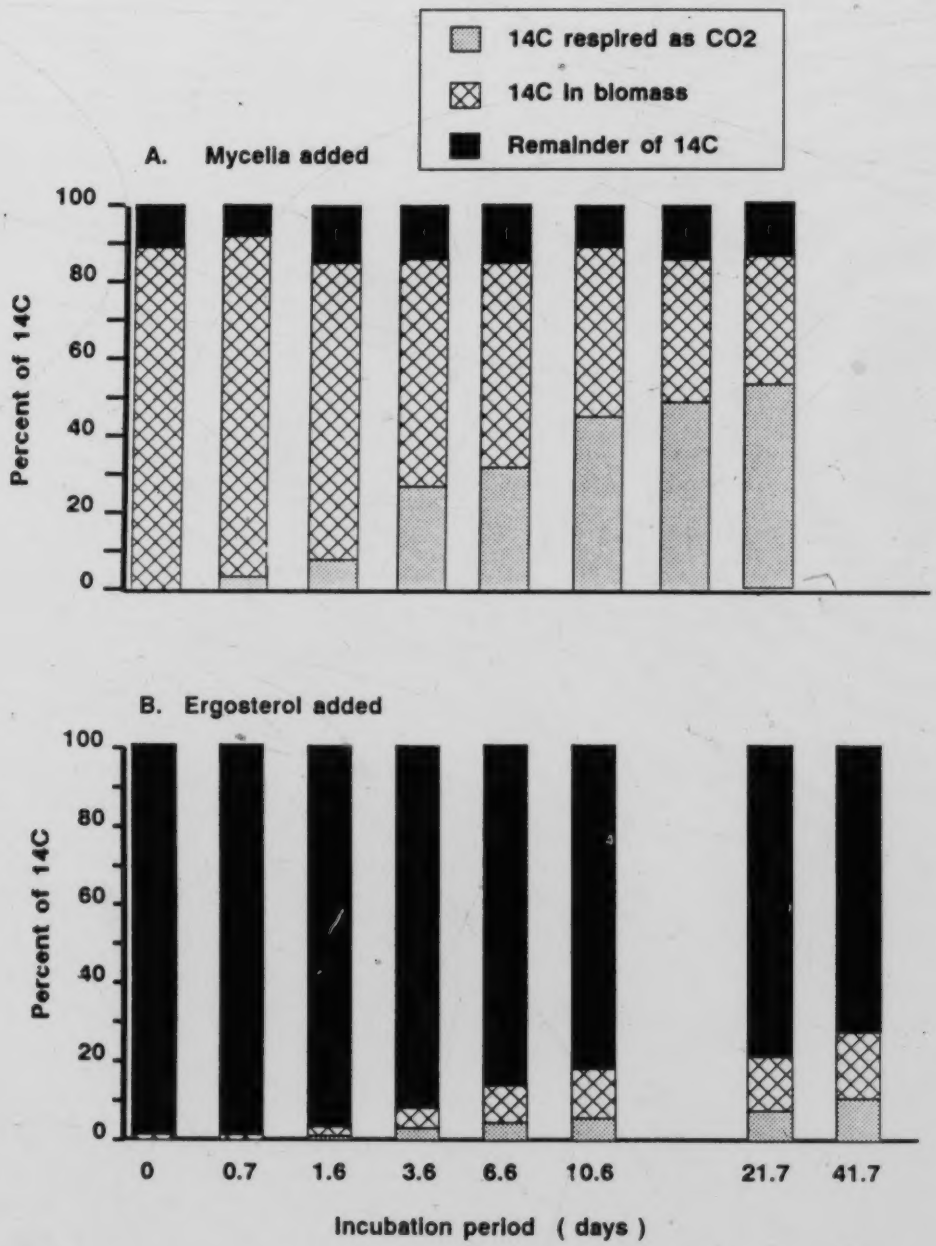

Figure 3.9. Distribution of 14C added to loamy sand as ergosterol or H. crustuliniforme mycelia.

CHAPTER 4

Use of ergosterol and chitin to estimate ectomycorrhiza establishment and *Pinus contorta* seedling response to inoculation[3]

4.1 Introduction

The extent of mycorrhiza formation on roots varies among fungal species (Shaw *et al.* 1982), plant cultivars (Graham and Syvertsen 1985) and soil fertility regimes (Maronek *et al.* 1981). Therefore, quantifying the effectiveness of mycorrhizal isolates in colonizing roots and enhancing plant performance requires determination of intramatrical mycelia mass. There are difficulties in interpreting morphology–based indices because mycelia mass is not determined; the number of ectomycorrhizal short roots or the percentage of the root with sheath development are commonly estimated (Molina and Chamard 1983; St. John and Hunt 1983).

Biochemical quantification techniques include: 1) the measurement of chitin (Hepper 1977); 2) fluorescein diacetate staining of active hyphae (Soderstrom 1979); and 3) analysis of ergosterol, a fungus–specific membrane component (Seitz *et al* 1979). The former two methods may be difficult to apply to mycorrhizal systems as they may be subject to soil interferences and significant portions of viable hyphae lost during sample preparation may not be enumerated (Bolker 1974; Soderstrom 1979; Parsons 1981). Ergosterol analyses have been reported for mycorrhizal roots (Salmanowicz and Nylund 1988), plants (Seitz *et al.* 1977) and soil (West *et al* 1987). Ergosterol is a minor component of most plants (Nes 1977).

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3. A version of this chapter will be submitted to Canadian Journal of Forest Research for publication. (Johnson, B.N. and W.B. McGill).

Ergosterol mass accounted for 77% of the variability in mycelia mass of *H. crustuliniforme* colonies (Chapter 2), but has not been compared to chitin as an estimate of intramatrical mycelia establishment. This led to the present study wherein roots of lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* engelm.) seedlings ectomycorrhizal with *H. crustuliniforme* were analyzed for ergosterol and chitin contents. Soil inorganic P status affects the extent of mycorrhiza establishment (Dixon *et al.* 1981; Frankland and Harrison 1985) and thus, influences of P fertilization regimes on root ergosterol and chitin concentrations were evaluated.

4.2 Objectives

The overall aim was to compare ergosterol and chitin analyses in quantification of intramatrical hyphae in seedling roots. Specific objectives were to:

1) determine mass of ergosterol and chitin in soil and pine seedlings as a function of inoculation of soil with *H. crustuliniforme* and soil P status;

2) determine if ergosterol and chitin analyses yield similar estimates of intramatrical mycelial mass; and

3) determine relationships among plant mass, inoculum addition and P addition.

4.3 Materials and Methods

4.3.1 Cultivation of mycelia

For the inoculation experiment, vegetative mycelia of the *H. crustuliniforme* isolate used in Chapter 2 were grown for 8 weeks in 2L-Mason jars filled with 900 mL finely sieved peat/vermiculite (1:14; v/v) and 550 mL Melin-Norkrans solution (Molina and Palmer 1982). Incubation was in the dark at 24 ^{o}C.

For determination of the chitin conversion factor, vegetative mycelia were grown for 50 days on cellulose acetate papers on complete Melin–Norkrans medium as in Chapter 2. Colony locations were sampled according to the procedure in Chapter 2 (section 2.3.2). Six replicated colonies were analyzed for chitin content.

4.3.2 Pine seedlot origin

Pinus contorta cones were collected from natural stands by the Alberta Forest Service. Seeds were extracted, cleaned and stored at the Pine Ridge Forest Nursery, Smoky Lake. The Nursery provided seeds from the following geographic range:

52.4 to 55.3^{O}N lat.,

115.2 to 120.0^{O}W long.,

870 to 1070 $\pm$ 20m elev.

4.3.3 Inoculation experiment

Air–dried subsamples of the soil previously described (Chapter 3) were autoclaved (1h; 120 ^{O}C) prior to being mixed with peat/vermiculite inoculum (1/9, v/v; inoculum/soil). For non–inoculated controls, autoclaved moist peat/vermiculite was used. Soil for each inoculation treatment was divided into three lots to receive KH_2PO_4–P at rates of 0, 50 or 100 mg kg^{-1} soil. Soil was then added to 1 L Spencer–Lemaire cavities to a bulk density of 1.0 Mg m^{-3} and moistened with sterile water (200 g kg^{-1}).

Lodgepole pine seeds were surface sterilized by a 15 min immersion in H_2O_2 (300 g L^{-1}) followed by 5 x 15 min rinses with sterile water. Seeds were germinated on agar plates and at 7 days, 2 seedlings were placed into the surface of each cavity. Trays of 18 seedlings were covered with plastic to minimize introduction of fungi indigenous to the glasshouse. Seedlings were grown in the glasshouse for 35 weeks with 17 hr daylengths, 22 ^{O}C days and 16 ^{O}C nights.

4.3.4 Sampling of plants

Aboveground plant parts were severed, dried at 60 ^{o}C for 18 hr and weighed. Roots were gently washed to remove mineral debris and fresh subsamples were analyzed for ergosterol and chitin contents. The remainder were dried and weighed.

4.3.5 Analytical biochemistry

Ergosterol assay The ergosterol assay described in chapter 2 was used. Root subsamples (up to 750 mg fresh mass) were added to test tubes containing 3.0 mL of anhydrous methanol with 0.14 g KOH and analyzed. Results were expressed as *u*g ergosterol g^{-1} dry root.

Chitin assay Chitin was assayed using a colorimetric procedure amended from Uchida and Yamaguchi (1984). In preliminary trials with varying KOH digestion times and temperatures, an 8 hr digestion at 130 ^{o}C yielded highest recovery (87 $\pm$ 5%) of purified chitin (Sigma Chemical Co., St. Louis, Mo.). Fresh roots (up to 750 mg) or mycelia (up to 300 mg) were refluxed in 12% KOH for 8 hr at 130 ^{o}C and cooled. Ice cold 75% ethanol (6 mL) was added and tubes centrifuged at 9750 x g at 4 ^{o}C for 20 min. Pellets were washed with cold 40% ethanol with 10% Celite 545, twice with cold water and resuspended in 1.5 mL of $NaNO_2$ solution (50 g L^{-1}) and 1.5 mL of $KHSO_4$ solution (50 g L^{-1}). The reaction mixture shaken for 15 min and centrifuged. To 2.0 mL aliquots of supernatents, 0.67 mL of ammonium sulfamate solution (125 g L^{-1}) was added, followed by 0.67 mL of MBTH (3-methyl-2-benzothiazolinone hydrazone) and then 0.67 mL of $FeCl_3$ solution (8.3 g L^{-1}). Samples were diluted with deionized water to remain within the working curve for determination of glucosamine (1 to 20 ug/mL). Glucosamine standards were similarly treated. After 25 min., the glucosamine equivalent was measured as optical density at 650 nm.

4.3.6 Statistical Analyses

The experiment comprised 3 fertilizer-P levels (0, 50 and 100 mg KH_2PO_4-P k^{-1} soil); and 2 inoculation treatments (+ or - *H. crustuliniforme*) as a factorial design replicated with 12 seedlings per fertilizer x inoculum combination. Analysis of variance was used to determine significance of treatments. Multiple means comparisons were performed according to Fisher's Least Significance Difference Method (Steel and Torrie 1980; Mize and Schultz 1985). The ergosterol assay involved fewer manipulations, hence, up to 4 times the number of samples could be prepared per day. Thus, means of root ergosterol concentration were represented by double the number of samples as chitin concentrations.

4.4 Results

4.4.1 Sources of variation in root ergosterol and chitin contents

Root ergosterol and chitin concentrations increased with addition of *H. crustuliniforme* mycelia ($p < 0.05$; Table 4.1). Chitin results were more variable than those for ergosterol (Figure 4.1); coefficients of variation ranged between 7 and 17% for ergosterol and 13 to 21% for chitin. Soil P fertilization altered root ergosterol but changes in chitin concentrations were not significant (Table 4.1). Mean root ergosterol concentration was highest in plants grown in non-fertilized soil (Figure 4.1). Among noninoculated roots, 50 and 100 mg P treatments contained 89 and 49% the amount of root ergosterol observed in the 0 mg case. For inoculated roots, 50 and 100 mg P treatments contained 60% the amount of the 0 mg P treatment. Mean root chitin concentrations of non-inoculated seedlings did not vary significantly among P treatments.

4.4.2 Estimation of intramatrical mycelial mass

Chitin concentration did not differ among locations within *H. crustuliniforme* colonies. Means of 6 replicates were 106.5 ± 19.1, 105.1 ± 14.9 and 104.7 ± 8.1 ug chitin mg^{-1} mycelia for colony core, midcolony and perimeter locations, respectively. Amino sugars from 12 week-old non-infected lodgepole pine roots grown gnotobiotically on agar yielded a colorimetric value equivalent to 3.13 ug glucosamine mg^{-1} dry root; this value was deducted from results of chitin assays. With root contributions subtracted, mycelia concentrations were estimated using 105.4 ug chitin mg^{-1} mycelia as a conversion factor. This value was previously shown to be constant in *H. crustuliniforme* mycelia of varying maturation states. Percent recovery of fungal chitin from roots was assumed to equal recovery of added chitin in preliminary trials.

Background levels of ergosterol in non-infected roots were negligible and thus mycelia mass in roots was estimated using the following equation derived for *H. crustuliniforme* pure cultures of varying ages and maturation states (Chapter 2):

$$Y = -5.46 + 0.38(X)$$

where, Y = mycelia mass (mg) and X = ergosterol mass (*ug*).

Ergosterol-based intramatrical mycelia was greatest at 0 mg P for both inoculation treatments (Figure 4.2). In the 100 mg P treatment, the decrease in root mycelia was 54% and 25% for non-inoculated and inoculated treatments, respectively. Chitin-based estimates of intramatrical mycelia mass were consisently lower than those derived from ergosterol. Ergosterol- and chitin-based estimates of intramatrical mycelia were not significantly correlated ($r = 0.15$). Ratios of ergosterol- to chitin-based mycelia mass estimates varied among fertilization treatments; as P fertility increased, the ratio decreased. At 0 mg P, inoculated and non-inoculated roots had ratios of 2.7 and 3.2, respectively compared to being 1.1 and 2.2 in the 100 mg P case.

4.4.3 Source of variation in plant performance

Root dry mass means averaged 70% higher in the inoculated treatments (Table 4.2; Figure 4.3) at all P treatments. Regardless of inoculation state, root mass means were not significantly affected by P fertilization. Total mass was increased 55 to 65% upon inoculation. Although 50 mg added P increased total plant mass of non-inoculated plants, the '0 P, non-inoculated' treatment was equal to *H. crustuliniforme* inoculation without P; inoculation alone could produce the same effect on plant yield as inoculation with fertilization.

Changes in plant growth and intramatrical mycelia upon inoculation were compared for the P treatments to determine the effectof inoculation on plant performance (Figure 4.4). Root mycelia mass, estimated using ergosterol increased in the 100 mg P treatment by a greater percentage than the increase in aboveground mass. A different plant growth response was observed in the 50 mg P case, where the root mycelia increased by only 17% upon inoculation and root and above-ground mass increase exceeded 50%.

4.5 Discussion

Differences among soil P treatments in extent of root infection were not distinquished by the chitin assay (Table 4.1, Figures 4.1 and 4.2). This may be attributed to small numbers of replications. Similarly, the greater relative technical ease and replication may have enhanced precision of the ergosterol assay. Effective indicators of fungal mass must be sensitive, reliable and time-efficient (Seitz *et al.* 1979). The ergosterol assay is less laborious per unit of replication. Lack of non-infected roots to account for root hexosamine contributions may be a further restriction of the chitin assay in field-grown plants.

Estimation of mycelia mass by chitin analysis may be made difficult by uncertainties in glucosamine recovery from intramatrical hyphae. Recovery of purified chitin was 87% which was higher than the 52% recovery reported by Plassard *et al.* (1982) but slightly lower than the 93% recovery obtained by Aidoo *et al.* (1981). Digestion in strong alkali was reported to reduce recovery from that of acid hydrolysis (Plassard *et al.* 1982) thus, digestion time and temperature must be optimized (Appendix A). Recovery of pure chitin may be higher than that of mycelial chitin integrated within a matrix of proteins and polysaccharides (Muzzarelli 1977; Gooday 1979), compounds that may interfere with recovery (Donald and Mirocha 1977). Nevertheless, chitin degradation during analysis, reported to be as high as 22% (West *et al.* 1987), may be minimized.

Hepper (1977) suggested that the chitin assay may be confounded by differences in expression of cell wall morphology in intramatrical and pure–cultured mycelia. If there are different morphological expressions of cell walls in the two systems, separate conversion factors for each must be obtained. It is not known if the present assumption of chitin content per mass of intramatrical mycelia satifies this criterion, although the conversion factor used here (105 *ug* chitin mg^{-1} mycelia) agrees with the value reported by Vignon *et al.* (1986) for *H. cylindrosporum* (107 *ug* chitin mg^{-1} mycelia). The use of the regression equation for estimating mycelial mass accounts for diverse ontogeny (Chapter 2) and thus may be applied to situations where diversity in maturation state is expected.

The chitin assay may be confounded by root–derived hexosamines (Vignon *et al.* 1986) but, ergosterol measurements may have greater analytical specificity (Matcham *et al.* 1985). Several sterols may be present in the root:fungal complex (Garroway and Evans 1984, Weete *et al.* 1985) but these can be separated from ergosterol by gas chromatography. With pure–culture studies of *Alternaria* sp. and *Aspergillus* sp., Seitz *et al.* (1979) observed observed ergosterol changes before chitin

changes were detectable. Matcham *et al.* (1985) also suggested that ergosterol analyses were less sensitive to chemical interferences. Thus, ergosterol may be a more sensitive index of mycelial mass.

The assays yielded similar qualitative conclusions about influences of *H. crustuliniforme* inoculation; both were sensitive to inoculation which increased mean ergosterol and chitin concentrations (Figure 4.1). Chitin-based mycelia mass estimates were less precise however (Figure 4.2), and the method failed, due to high variability, to distinguish differences that are demonstrable with ergosterol. Further, the constitution of wall and membrane components may undergo changes at different rates or to a different extent (Paustian and Schnurer 1987). This may have contributed to the weak correlation of ergosterol and chitin contents. At low substrate levels, chitin accumulation in intramatrical mycelia was lower than that of ergosterol, while at high soil P fertility, the rate of chitin accumulation increased relative to that of ergosterol to yield higher ergosterol- / chitin-based ratios in the 0 mg P treatment.

Differences in ergosterol-based mycelia concentration may be used to interpret influences of fungi on plant growth among P treatments. Root ergosterol content was influenced by soil P treatment (Figure 4.1) but root growth was not (Figure 4.3). The P effect on infection conforms to earlier reports of reductions in mycorrhizae with increasing soil P fertility (Sanders 1975). The reduction in root ergosterol content with high P fertilization, coupled to the lack of change in root growth (Figure 4.4) suggests that a unit amount of intramatrical hyphae may sustain a greater level of root growth at high soil P contents. The introduced fungus in the 100 mg P case is evidently lower in 'efficiency', as a large increase in plant growth did occur along with the large % increase in intramatrical hyphae.

Carbon sink strength in plants may be altered upon mycorrhizal infection (Trappe 1977); thus, there is a need to assess plant growth response to a unit amount of fungal tissue residing within roots. Quantification of extramatrical mycelia would be desired as an indicator of the strength of the mycobiont as a carbon sink. Detection of glucosamine in soil would include amino sugars of non-fungal origin, (e.g. insects) which may have extended residence times in soil (Parsons 1981). This has led some authors (West *et al.* 1987; Sharma *et al.* 1977) to regard the chitin assay as being of limited use in soil. Ergosterol may be a suitable biochemical marker to fulfill this need.

4.6 Conclusions

Ergosterol is a practical parameter to monitor in mycorrhizal roots; the assay for this compound has specificity and sensitivity advantages over that for chitin as roots are ergosterol-free and do not have interfering compounds. Ergosterol and chitin contents of roots do not provide similar estimates of intramatrical mycelial mass; physiologic or morphologic origins of such differences have not been resolved. In the case of *P. contorta* ectomycorrhizal with *H. crustuliniforme*, interactions of inoculum and P fertilization were not apparent in seedling mass averages. Seedling root and total masses after 35 weeks of growth are affected by inoculation with *H. crustuliniforme*, while inorganic P fertilization did not affect mass accumulation.

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Table 4.1. F ratios and error mean squares of analysis of variance for root ergosterol and chitin concentrations of 35 week-old pine seedlings.

Source	d f	Dependent variable: Ergosterol	Chitin
		F values	
Inoculation (I)	1	15.92**	20.08**
P fertilization (P)	2	5.14**	0.34
I X P	2	1.26	0.88
		Mean Squares	
Error	65 , 30*	0.117	0.099

* total of 71 ergosterol analyses, 36 chitin analyses
** F ratios are significant at $P < 0.05$.

Table 4.2. F ratios and error mean squares of analysis of variance for root mass and total mass of 35 week-old pine seedlings.

Source	d f	Dependent variable: Root mass	Dependent variable: Total mass
		F values	
Inoculation (I)	1	34.67**	94.65**
P fertilization (P)	2	1.72	7.28
I X P	2	0.32	2.71
		Mean Squares	
Error	65*	15592	13644

* total of 71 analyses.
** F ratios are significant at $P < 0.05$.

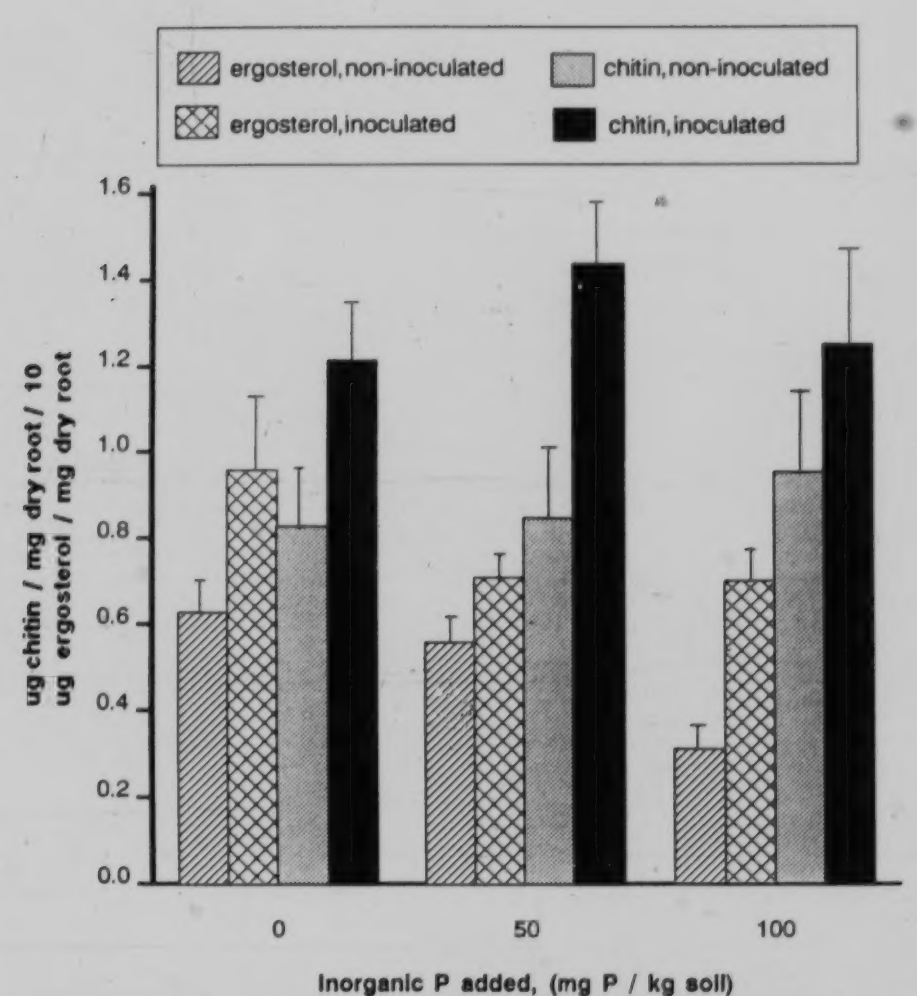

Figure 4.1. Ergosterol and chitin concentrations of 35 week-old pine roots grown with or without H. crustuliniforme or inorganic-P addition. Bars indicate standard errors of means. For ergosterol, n=12, except for '0 P,non-inoculated' treatment where n = 11; for chitin, n = 6.

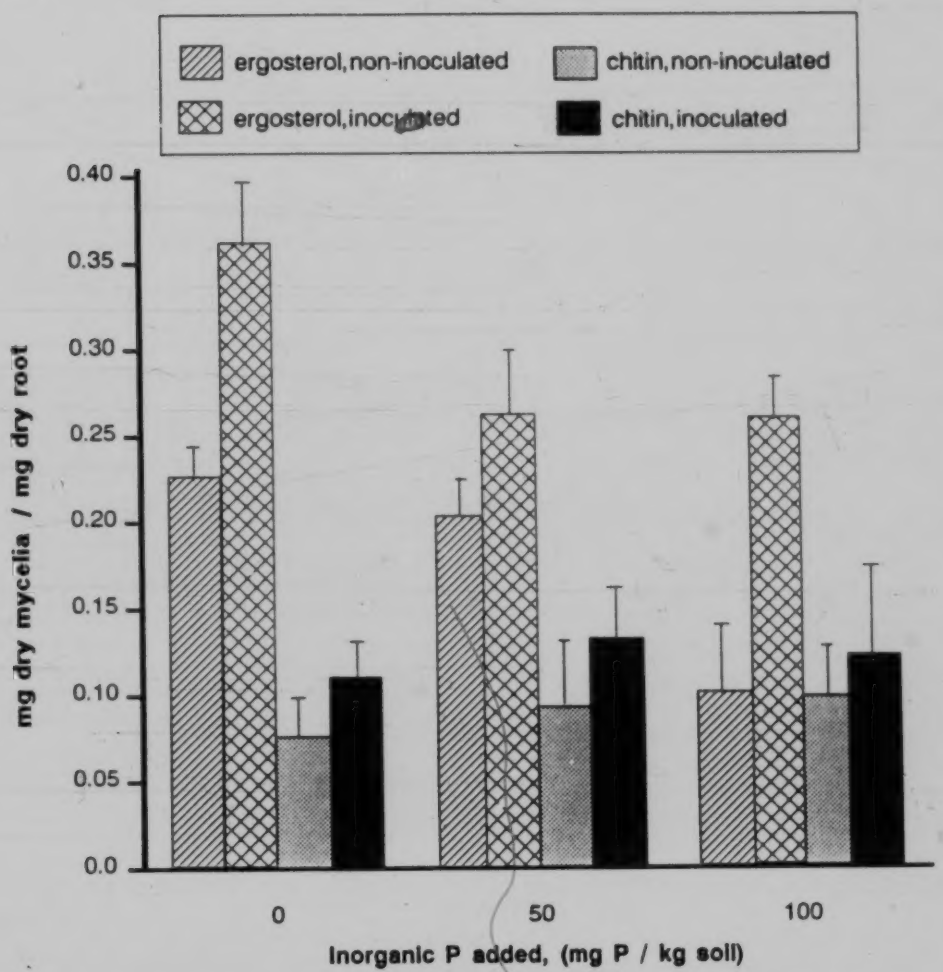

Figure 4.2. Estimated intramatrical mycelia contents of 35 week-old old pine roots grown with or without H. crustuliniforme or inorganic P addition. Estimates based on ergosterol and chitin concentrations. Bars indicate standard errors of means.

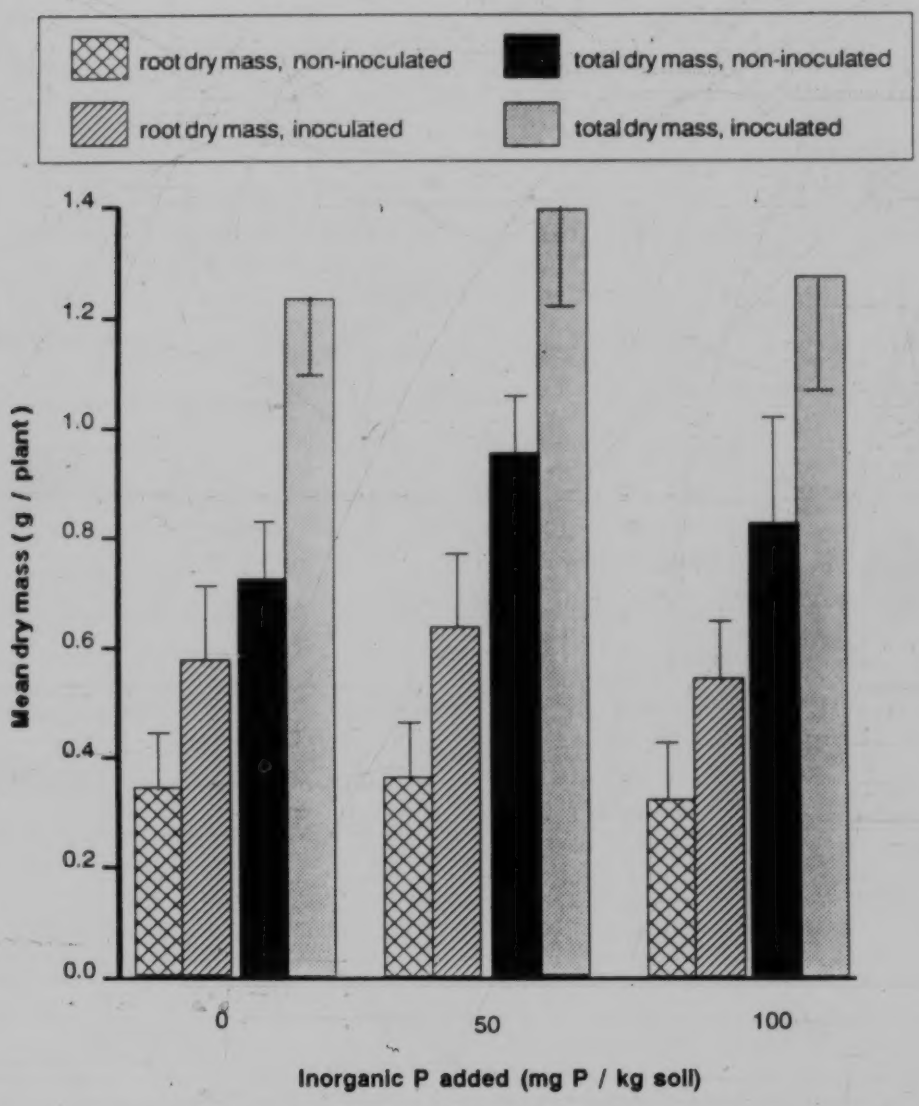

Figure 4.3 Total mass and root mass of 35 week- old pine seedlings grown with or without inorganic-P addition. Bars represent standard errors of means.

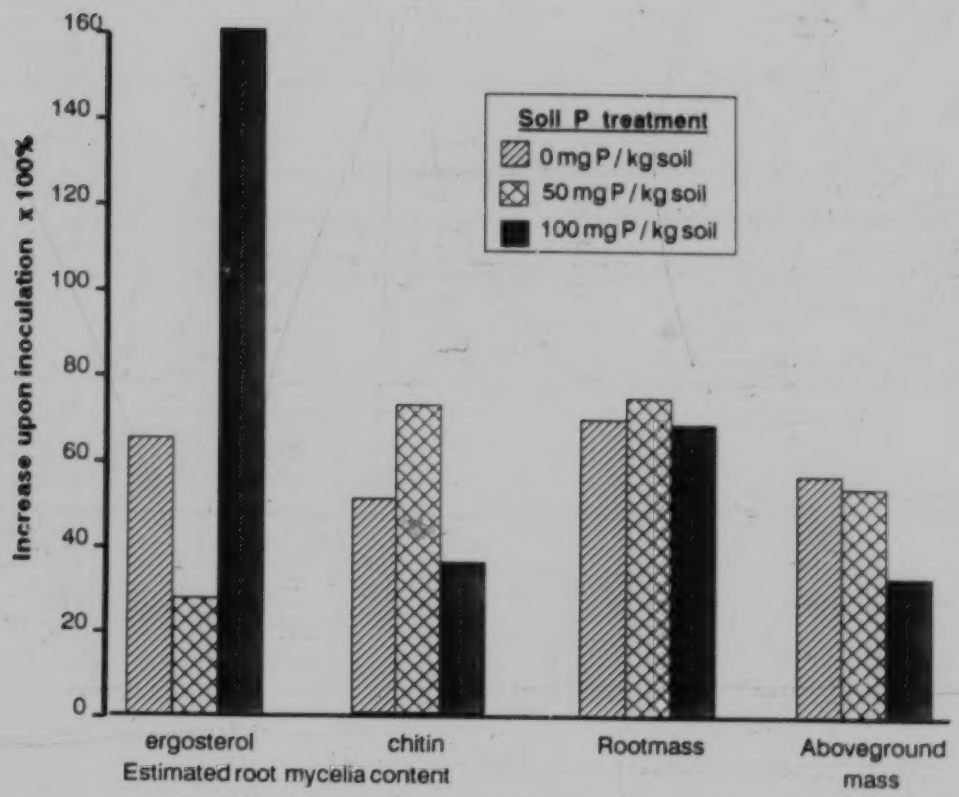

Figure 4.4. Root mycelia and seedling mass increases upon addition of H. crustuliniforme or inorganic-P.
Values calculated as (inoculated - noninoculated) / (noninoculated).

CHAPTER 5

Spatial and temporal variations in ergosterol content and ornithine decarboxylase activity of ectomycorrhizal *Pinus contorta* root systems[4]

5.1 Introduction

The enhancement of microbial activity and biomass in the rhizosphere has been well established. As much as 40% of photosynthetic-C may be exuded by roots (Barber and Martin 1976, Newman 1978) and microbial biomass along roots may be 200% higher than in soil some distance away (Helal and Sauerbeck 1983). The rhizosphere effect has commonly been examined in root systems of cereals and other crops associated with vesicular-arbuscular mycorrhizae (Smith 1980).

Ectomycorrhizal fungal symbionts are also thought to modify rhizosphere characteristics and composition (Rambelli 1973). Ectomycorrhizal hyphae expend large fractions (up to 25%) of fixed-C (Newman 1978) and represent as much as 40% of root mass (Harley and McCready 1952). They may affect exudate quantity; Krupa and Fries (1971) recorded an increase in exudation of up to eightfold by mycorrhiza establishment in *Pinus sylvestris* roots. Ectomycorrhizal hyphae are also known to produce hormones (Slankis 1973) that affect whole plant physiology. These reports suggest that the introduction of ectomycorrhizal fungi may have a significant impact on the composition and metabolic activity of the microbial community along roots however, quantitative descriptions of such influences have not been documented.

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4. A version of this chapter will be submitted to Plant and Soil for publication. (Johnson, B.N. and W.B. McGill).

Plant age may also affect rhizosphere ecology by influencing root exudation. Bowen (1969) documented a decline in amino acid content of root exudates of *Pinus radiata* seedlings from 2 to 6 weeks. In a study with several grasses, Dommergues *et al.* (1973) reported an increase in the rhizosphere effect as plants matured, with a peak reached at the height of vegetative development and a decline as roots senesced. Temporal changes in rhizosphere activity of ectomycorrhizal roots have not been reported, nor has there been an attempt to describe biochemical features of intramatrical hyphae during the ontogeny of mycorrhization.

Levels of soil nutrients affect the magnitude of mycorrhiza establishment and growth response of the host (Smith 1980). Effects of phosphorus deficiency on ectomycorrhizae have been extensively examined for influences on tree seedling performance in reforestation schemes. The scarcity of soil P permits ectomycorrhiza establishment to begin earlier and at a faster rate than when P is not limiting (Maronek *et al.* 1981). Turner *et al.* (1985) suggested that P deficiency increases root exudation in perennial ryegrass and proposed that, with other nutrients also in short supply, rhizosphere fungi may be more competitive. There is no information on effects of soil P on cumulative establishment of intramatrical hyphae, nor is there evidence for an effect of soil P on fungal mass and biotic activty in ectomycorrhizal root–soil systems.

Existing information regarding ectomycorrhizal symbioses has led to the present study wherein *Pinus contorta* seedlings were grown with or without the addition of *H. crustuliniforme* in a P–deficient soil amended at different P fertilization rates. Although Bennett and Lynch (1981) cautioned that studies of soil are difficult to reproduce, they are necessary to understand synecological features of the rhizosphere. Biochemical markers were judged to be informative when used with soils and roots (Chapters 2, 3 and 4) and were used here to monitor mass/activity relations of mycorrhizal roots.

Earlier investigations with pure-cultured *H. crustuliniforme* (Chapter 2) provided a conversion factor to estimate mycelial mass from ergosterol content. Ornithine decarboxylase activity is linked to polyamine biosynthesis potential (Walters *et al.* 1985). Activity of this enzyme was found to be correlated with growth capacity of the fungus (Chapter 2) and was used here to monitor metabolic activity of roots, rhizosphere soil and nonrhizosphere soil

5.2 Objectives

Influences of soil chemical amendments and temporal aspects of rhizosphere ecology in mycorrhizal root:soil systems were quantified. The specific objectives were to:

1) determine if inoculation of *Pinus contorta* rooting medium with *H. crustuliniforme* influences ODC activities or mycelia contents of bulk soil, rhizosphere soil and roots during early phases of seedling growth.

2) determine if soil P fertilization influences on ODC activities or mycelia content of bulk soil, rhizosphere soil and roots within the first year of seedling establishment.

3) determine if inoculation or P fertilization affects seedling performance within the first year of establishment.

5.3 Materials and Methods

Vegetative mycelia of *H. crustuliniforme* were grown in peat/vermiculite as described in Chapter 4. Loamy sand described in Chapter 3 was ground to pass through a 2 mm sieve, amended with fungi-enriched or autoclaved peat/vermiculite (1:9, v/v; inoculum/soil) and moistened (200 g kg^{-1} soil) prior to planting.

Pinus contorta seeds representing one seedlot (53.0°N lat., 115.2°W long.; 940 ± 20 m; collected May, 1981) were supplied by the Pine Ridge Forest Nursery, Smoky Lake, Alberta. Seeds were surface-sterilized with 30% H_2O_2 for 5 minutes, rinsed 5 times with sterile water, germinated and seedlings grown on agar for 7 days prior to planting.

5.3.1 Growth experiments

Ontogeny of mycorrhization.

The experiment comprised 2 treatments (with or without *H. crustuliniforme*), 3 sampling dates for roots and rhizosphere soils (6, 9 and 12 weeks) and 4 for bulk soil (week 0 included). Sterile or fungus-enriched peat/vermiculite was mixed into air-dried autoclaved (120 °C; 60 min) soil (1 part amendment:9 parts soil; v/v). Soil was deposited into 0.75 L Spencer-Lemaire containers and compressed to a bulk density of 1 Mg m^{-3}. Each container held one seedling and was covered with plastic and placed in the glasshouse (17 hr daylengths; 22 °C days; 16 °C nights). At each sampling date, 12 seedlings from each treatment were harvested and combined to represent 4 replicates for each date x treatment combination. Rhizosphere and non-rhizosphere soils were similarly composited.

Influences of P fertilization on rhizosphere ecology.

A second experiment was comprised of 2 fertilizer treatments (no P added; 50 g KH_2PO_4-P kg^{-1} dry soil); 2 inoculation treatments (*H. crustuliniforme*; control); and 2 sampling dates (15 and 21 weeks) with three replications of each fertilizer x inoculation x date combination. Soil preparation and bulk density and growth conditions were as in the first experiment except that seedlings were raised in 0.20 L glass tubes. Tubes were covered with plastic and placed in racks in the glasshouse. In both experiments, soil water was maintained at 150 to 200 g kg^{-1} soil.

5.3.2 Sampling of plants

Aboveground portions of seedlings were severed at the root collar, dried at 60 ^{o}C for 18 hr and weighed. Roots were removed by inverting glass tubes and allowing intact soil cores to slide out or by cutting containers along the side. Material adhering to roots, taken to represent the rhizosphere, was gently scraped from the roots and processed separately from the non-rhizosphere soil that fell away readily. Roots and rhizosphere soils were processed for ODC activity and the residues further processed for ergosterol content. Ornithine decarboxylase and ergosterol analyses of non-rhizosphere soils were performed on separate subsamples.

5.3.3 Analytical biochemistry

Assay of ODC activity.

The assay procedure described in Chapter 2 was followed using approximately 250 mg of fresh roots or 500 mg of fresh soil. Enzyme activities were expressed as pmols/hr per g soil; pmols/hr per g root and pmols/hr per mg mycelia.

Ergosterol assay.

The ergosterol procedure described in Chapter 2 was followed. Fresh roots (up to 750 mg) and soils (up to 5 g) were added to test tubes containing 3.0 mL (for roots and rhizosphere soils) or 6.0 mL (for non-rhizosphere soils) anhydrous methanol with 0.14 g KOH per 3 mL. Mycelia mass was calculated by the following regression equation derived for *H. crustuliniforme* colonies:

$$Y = -5.46 + 0.38(X)$$

where Y = mycelia mass (mg) and X = ergosterol mass (μg).

5.3.4 Statistical Analyses

Fisher's Least Significance Difference Method was applied to assess significance of differences among inoculation and fertilization treatments (Steel and Torrie 1980; Mize and Schultz 1985). Standard errors of means were also calculated.

5.4 Results

5.4.1 Ornithine decarboxylase activity of roots and soils

Addition of *H. crustuliniforme* inoculum increased ODC activity of roots at all sampling times (Figure 5.1). For both inoculation treatments, root ODC activity declined over time, with 21-week values nearly 25% of 6-week values. Rhizosphere ODC activity increased with inoculation (Figure 5.2); 20 to 50% increases were observed. Temporal decreases in rhizosphere activity were observed and were of the same magnitude for inoculated and noninoculated roots. Comparing 6- to 21-week values, rhizosphere ODC activity decreased by 75%.

Bulk soil ODC activity expressed on a dry mass basis was 2.4 fold greater in the inoculum-enriched soil at time zero (Figure 5.2). The inoculum effect in bulk soil lessened over time; activity was 70% higher at 6 weeks and 54% at 12 weeks. By 15 and 21 weeks, bulk soil activities did not differ. The temporal decrease in inoculated bulk soil activity was greater than that of non-inoculated soil. Twelve-week activity of inoculated bulk soil was 38% of the initial value, compared to a retention of 71% of initial activity in the non-inoculated case.

Comparison of soil components reflects spatial variability in ODC activity (Figures 5.1 and 5.2). Rhizosphere activity was 4 to 5 times greater than bulk soil activity within 12 weeks. By 21 weeks, bulk soil activity was one-third to one-half that of the rhizosphere.

Fertilizer-P produced a positive effect on root and rhizosphere ODC activity at 15 weeks. In rhizosphere soil, the influence was most pronounced with inoculum added; activities of the '+H' and '-H,' treatments were 100% and 65% higher than those of respective '-P' treatments (Figure 5.3).

5.4.2 Mycelia concentrations of roots and soils

Roots from fungi-enriched soil had greater mycelia concentration at all sampling times (Figure 5.4); intramatrical mycelia mass per unit mass of inoculated roots was 25% higher at 6 weeks, 70% at 9 weeks and 2.1 fold greater at 12 weeks. Mycelia concentration of non-inoculated roots declined by 84% from 6 to 12 weeks, while the temporal decrease in inoculated roots was 60%. From 12 to 21 weeks, mycelia content increased by 20 to 30% for both inoculation treatments.

Relative differences in mycelia rhizosphere content among inoculation treatments changed as the experiment progressed; inoculation increased mycelia content by 8, 56 and 71% at 6, 9 and 12 weeks, respectively (Figure 5.5). Rhizosphere mycelia content of inoculated soils did not exhibit a distinct trend within 12 weeks but declined by 50% by 21 weeks. A gradual decrease in rhizosphere and bulk soil mycelia concentration was also observed over the growth period (Figure 5.5).

Bulk soil with added inoculum contained 2 times more mycelia mass than the non-inoculated soil at time 0 (Figure 5.5). By 6 weeks, mycelia masses were nearly equal. At 15 weeks, inoculation increased abundance of mycelia in bulk soil but the magnitude of the increase depended upon P status (Figure 5.6). Without PO_4-P, inoculation increased bulk soil mycelia by 2 fold, with only 25% with PO_4-P added.

Spatial differences in mycelia content, expressed on an equal mass basis were apparent. Roots were enriched with more mycelia than rhizosphere soil (Tables 5.1 and 5.2), with greatest differences at the earliest date. For the inoculated system, differences at 6 and 12 weeks were 6 and 1.3 fold, respectively. For non-inoculated systems, roots harbored 4 times more mycelia at 6 weeks, but near equal amounts at 12 weeks. At 15 weeks (Figure 5.6), rhizosphere mycelia contents were 2 to 4 times higher than corresponding bulk soils.

5.4.3 ODC activity as a function of mycelia concentration

In bulk soil, the greatest activity when expressed on a mycelia mass basis was observed in the '-H,-P' treatment (Figure 5.7). Additions of *H. crustuliniforme* and P fertilizer reduced ODC activity expressed on this basis. In the rhizosphere, P addition increased mycelia-specific ODC activity from the respective '-P' cases. Inoculum addition resulted in less pronounced increases in rhizosphere values, although the highest mean value was obtained in the rhizosphere of the '+H,+P' treatment. Mycelia-specific ODC activity increased with the addition of *H. crustuliniforme* in both fertility regimes.

5.4.4 Influences of inoculation and fertilization on plant performance.

Addition of *H. crustuliniforme* to soil decreased mean seedling root mass within 21 weeks of growth (Table 5.3). For non-fertilized soils, the reduction was 48% and 27% at 15 and 21 weeks, respectively. For fertilized soils, root masses of inoculated seedlings were 63 and 35% lower at the same times. Fertilization did not enhance root mass averages within 21 weeks however, from a previous experiment (Chapter 4), inoculation increased root mass per seedling by 35 weeks.

The effect of inoculation on shoot mass averages was consistently positive in soil amended with P (Table 5.4). However, shoots of 21 weeks old seedlings in non-fertilized soil decreased 20% with inoculation. The effect of fertilizer-P at 21 weeks was dependent upon soil inoculation state. With inoculum added, P fertilization increased shoot mass by 21% but without inoculation, a decrease of 20% was observed. By 35 weeks, inoculation had produced plants with lower shoot:root mass ratios (Table 5.5). Among the 15- and 21-week seedlings, fertilizer-P increased shoot:root ratios of inoculated plants only.

Inoculation increased the total ODC activity of roots, especially at early times and in the absence of fertilizer-P (Table 5.6). Values for this parameter increased from 6 to 15 weeks and then declined by an average of 20% at 21 weeks. A similar temporal pattern was evident in rhizosphere ODC activity (Table 5.7), with maximum total rhizosphere ODC activity observed at 12 weeks, with declines of 80 to 85% by 21 weeks.

From 6 to 15 weeks, total root mycelia mass remained relatively constant and increased sharply from 15 to 21 weeks (Table 5.1). Further increases in the total intramatrical mycelia content of seedlings was observed by 35 weeks. Total root mycelia increased from 15 to 21 weeks in all treatments except the '-H,-P' treatment. Inoculation increased total mycelia mass in 21-week old roots of fertilized soils but in non-fertilized soils, the abundance of root mycelia was equal.

The total mass of rhizosphere mycelia per seedling was greater with seedlings from the inoculated condition after 12 weeks, (Table 5.2). At this time and at 21 weeks, P fertilization did not have a significant effect on rhizosphere mycelia mass. Inoculation also increased the mass of soil adhering to a unit mass of roots while fertilization did not; inoculated seedlings harbored 2 to 3 times more mycelia in the rhizosphere soil volume than non-inoculated plants. Total rhizosphere mycelia mass decreased slightly from 15 to 21 weeks.

The ratio of root:rhizosphere mycelia mass varied. Non-inoculated seedlings generally had a higher ratio than inoculated plants in the same fertilizer treatment. For '-H,-P' and '-H,+P' seedlings at 15 weeks, the ratios were 1.7 and 1.4, compared to 0.4 and 0.7 for '+H,-P' and '+H,+P' seedlings, respectively.

5.5 Discussion

Although autoclaved prior to planting, non-inoculated soils were not sterile at the first sampling time; ergosterol was detected in roots at this time (Figure 5.4) and at higher levels than in non-inoculated *P. sylvestris* roots grown in a sand:perlite:vermiculite composite (Salmanowicz and Nylund 1988). In preliminary trials with agar-cultured *P. contorta* roots, ergosterol was not detected and thus, ergosterol analyses here detected either indigenous fungal species that germinated from viable propagules within soil, or species introduced as aerial contaminants. Such fungi appeared to be competent mycobionts with *P. contorta*. Histochemical examination of roots would be needed to attribute increased ergosterol to the introduced strain. There is no basis presently for assuming that *H. crustuliniforme* merely enhanced colonization opportunities for other potential mycobionts while not establishing its own intramatrical mycelia.

Addition of vegetative mycelia of the ectomycorrhizal fungus affected rhizosphere ecology and root metabolism in both experiments, increasing ODC activity and mycelia concentrations in *P. contorta* roots and rhizosphere soil (Figures 5.1 to 5.5). The introduced strain was apparently a competent colonizer of rhizosphere soil and roots as inoculation generally increased rhizosphere and root mycelia content (Tables 5.1 and 5.2).

A relatively minor presence of intramatrical mycelia was observed in the first 12 weeks with greater fungal mass after 21 weeks (Figure 5.4 and Table 5.1); this indicates that rapid establishment of intramatrical hyphae did not occur until after 12 weeks. Littke *et al.* (1980) documented *H. crustuliniforme* colonization of 20% of Douglas-fir root systems within 2 weeks. A slower rate of mycorrhization with *P. contorta* is indicated here. Establishment of 125 mg mycelia per g root (Figure 5.4) is 3 times higher than *H. crustuliniforme* infection levels on similarly aged *P. sylvestris roots* (Salmanowicz and Nylund 1988). This discrepancy may be due to host differences in susceptability to *H. crustuliniforme* establishment, differences in growth conditions of vitality of the inoculum.

Inoculation of soils with *H. crustuliniforme* resulted in decreased root mass early in the experiments, but yielded benefits to plants by 35 weeks (Table 5.3). Such a transitory decrease may be dependent upon ontogeny of the plant and the mycobiont. Lowering of root mass and raising of the shoot:root ratio was described by Smith (1980) as a result of a fast growing endophyte and low light intensity combining to alter source–sink relations within the plant. These experiments were conducted during summer months with lighting supplements and thus, photosynthate availability was not likely to have been limiting. Also, while inoculation increased shoot:root mass at varying times (Table 5.5), an influence on total root ODC activity was not apparent. The significance of root ODC activity as an indicator of source–sink relationships remains unclear.

The enhancement of root ODC activity upon mycorrhiza establishment is consistent with changes in polyamine biosynthesis enzyme activities observed by Walters *et al.* (1985) upon fungal infection of barley leaves. Smith *et al.* (1985) also reported fungus–enhanced activities in glutamate dehydrogenase and glutamine synthetase in roots. Maintenance of the mycobiont may have constituted a portion of the increased root activity.

Temporal changes in rhizosphere ecology and root metabolism at early stages of pine seedling development are evident. The decline in root and rhizosphere ODC activity (expressed on a dry mass basis) from 6 to 21 weeks (Figures 5.1 and 5.2) may be caused by developmental changes in roots; by 21 weeks, a lower proportion of the root mass may be involved in exudation processes. Zones of maturation are not as active in active and passive exudation processes as apical zones (Smith 1980). Thus considering the entire root system, the availability of plant–derived substrates in the rhizosphere may diminish with time.

The different temporal patterns in total rhizosphere ODC activity and mycelia content (Tables 5.1, 5.2, 5.6 and 5.7) may reflect altered population structure at varying periods of root development. For example, at 21 weeks, total rhizosphere mycelia content of inoculated seedlings in the fertilized soil was 10 times the value at 6 week, while total rhizosphere ODC activity was nearly equal at the two times. Similarly, total root activity remained relatively unchanged while mycelia content increased three fold. The rhizosphere has been reported to be dominated by bacteria while fungi predominate in non–rhizosphere soils (Sparling 1985). Although mycorrhizal structures alter rhizosphere community composition (Marx and Krupa 1978), quantitative studies wherein rhizosphere biology is manipulated would increase understanding of mycorrhiza influences on root metabolism and soil ecology.

Spatial differences in ODC activity were apparent among the three sampled components. Roots harbor 10 times more ODC activity on a mass basis than soils. This is not unexpected, as soils are relatively devoid of biologically viable tissue. However, on an equal mass basis, mycelia are much more active than roots. Maximal fungal activity in pure culture conditions was approximately 10 times greater than the highest mean for roots. Much of the root mass is structural or conducting tissue and thus may not be high in ODC activity.

Determination of ODC activities of soil components has indicated that chemical and biological amendments influence soil microbiota. The '-H,-P' and '-H,+P' treatments had similar root ODC activities at 15 weeks (Figure 5.3) and thus, the rooting medium lacking P may not have imposed a nutritional stress on seedlings. Non-inoculated root ODC activities were not significantly altered by soil P fertilization and hence, soil P had no discernable effect on root metabolism.

High rhizosphere ODC activity and mycelia content in '+H,+P' treatment at 15 weeks (Figures 5.3 and 5.6) suggest that high P status permits a large community dominated by extramatrical hyphae to be sustained in the mycorrhizosphere. The markedly low rhizosphere activity in the '+H,-P' case at 15 weeks, coupled to high root activity, implies that mycorrhizal structures limit the activity of rhizosphere biota if inorganic P is scarce. Bowen (1969) observed that P deficiency increased amino acid and amide exudation in *P. radiata* roots but, extensive mycorrhization may have inhibited any leakage here.

Expressions of mycelia-specific ODC activity (Figure 5.7) demonstrated that in bulk soil, less of the activity can be attributed to fungi with addition of *H. crustuliniforme*. Also, in the absence of *Hebeloma*, addition of P greatly reduces fungus-specific activity. In this latter case, bacterial activity may be a major contributor to ODC activity. In comparison, the rhizosphere displays greater sensitivity in fungus-specific activity with the addition of P.

5.6 Conclusions

H. crustuliniforme inoculation increased rhizosphere mycelia mass and *Pinus contorta* root ODC activity. Phosphorus fertilization had no effect on root ODC activity, but increased rhizosphere ODC activity. Intramatrical mycelia establishment proceeded slowly within the first 15 weeks of planting. Within 15

weeks of planting, inoculated seedlings had lower mean root masses. By 35 weeks, the effect of inoculation on plant growth was positive and quanitity of intramatrical mycelia was one order of magnitude higher than the value at 21 weeks. Thus, mycorrhizal root:fungi interactions exhibited temporal dynamics that may be resolved using ergosterol and ODC activity.

Activity of ODC in the rhizosphere was affected by inoculation and fertilization, with interactions among the two factors governing activity; ODC activity in the rhizosphere was greatest with mycorrhizal fungi and P added together. Rhizosphere soil activity declined with time.

Bulk soil, rhizosphere soil and roots display different changes in mycelia content due to inoculation and P fertilization. A greater quantity of fungal mass is supported in the rhizosphere, compared to bulk soil. It is not known whether or not such a difference is maintained over longer periods of plant growth. Rhizosphere mycelia abundance does not appear to be controlled by P status as PO_4-P fertilization had no effect on mycelia estimates, while inoculation had a positive effect.

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Table 5.1. Total intramatrical mycelia mass of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	mg intramatrical mycelia / seedling *					
	Sampling time (weeks)					
Treatment	6	9	12	15	21	35
- H, - P				6.4 *b*	4.5 *a*	
+ H, - P				3.8 *a*	5.0 *a*	
- H, + P	2.8 *a*	2.3 *a*	1.9 *a*	4.3 *a*	11.1 *b*	73 *a*
+ H, + P	4.3 *a*	3.6 *a*	3.5 *b*	4.3 *a*	14.8 *b*	165 *b*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

Table 5.2. Total rhizosphere mycelia mass of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	mg rhizosphere mycelia / seedling *				
	Sampling time (weeks)				
Treatment	6	9	12	15	21
- H, - P				3.1 *a*	2.4 *a*
+ H, - P				7.8 *b*	7.3 *b*
- H, + P	0.7 *a*	2.0 *a*	2.7 *a*	3.5 *a*	2.1 *a*
+ H, + P	0.7 *a*	2.8 *a*	4.8 *b*	7.9 *b*	7.2 *b*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

Table 5.3. Root dry mass of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	mg root mass / seedling *					
	Sampling time (weeks)					
Treatment	6	9	12	15	21	35**
- H, - P				126.1 *b*	144.2 *ab*	
+ H, - P				85.0 *ab*	113.6 *a*	
- H, + P	23.4 *a*	50.4 *a*	95.6 *b*	108.2 *b*	159.9 *b*	365 *a*
+ H, + P	28.6 *a*	45.1 *a*	57.3 *a*	66.5 *a*	117.6 *a*	635 *b*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).
** data previously reported (Chapter 4).

Table 5.4. Shoot dry mass of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	mg shoot mass / seedling *					
	Sampling time (weeks)					
Treatment	6	9	12	15	21	35**
- H, - P				60.8 *ab*	115.0 *b*	
+ H, - P				71.2 *b*	92.7 *ab*	
- H, + P	31.2 *a*	43.3 *a*	46.4 *b*	55.4 *a*	86.8 *a*	592 *a*
+ H, + P	46.5 *a*	47.6 *a*	58.5 *a*	73.2 *b*	112.1 *b*	759 *b*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).
** data previously reported (Chapter 4).

Table 5.5. Ratio of shoot:root mass of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	Shoot / root ratio *					
	Sampling time (weeks)					
Treatment	6	9	12	15	21	35
- H, - P				0.51 *a*	0.80 *b*	
+ H, - P				0.94 *b*	0.83 *b*	
- H, + P	1.35 *a*	0.86 *a*	0.48 *a*	0.52 *a*	0.54 *a*	1.62 *b*
+ H, + P	1.64 *a*	1.04 *a*	1.02 *b*	1.11 *b*	1.00 *b*	1.14 *a*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

Table 5.6. Total root ODC activity of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	Root ODC activity (pmol / h / seedling) *				
	Sampling time (weeks)				
Treatment	6	9	12	15	21
- H, - P				22.7 *a*	21.0 *ab*
+ H, - P				27.1 *a*	28.6 *b*
- H, + P	11.6 *a*	17.8 *a*	22.5 *a*	21.9 *a*	17.3 *a*
+ H, + P	18.9 *a*	19.9 *a*	19.3 *b*	24.0 *a*	17.1 *a*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

Table 5.7. Total rhizosphere ODC activity of pine seedlings grown with or without H. crustuliniforme inoculation or inorganic-P fertilization.

	Rhizosphere ODC activity (pmol / h / seedling) *				
	Sampling time (weeks)				
Treatment	6	9	12	15	21
- H, - P				13.0 *a*	3.8 *a*
+ H, - P				35.1 *ab*	6.6 *ab*
- H, + P	7.3 *a*	16.0 *a*	14.3 *a*	23.6 *a*	3.4 *a*
+ H, + P	7.5 *a*	18.0 *a*	23.3 *b*	60.8 *b*	8.6 *b*

* within each sampling time, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

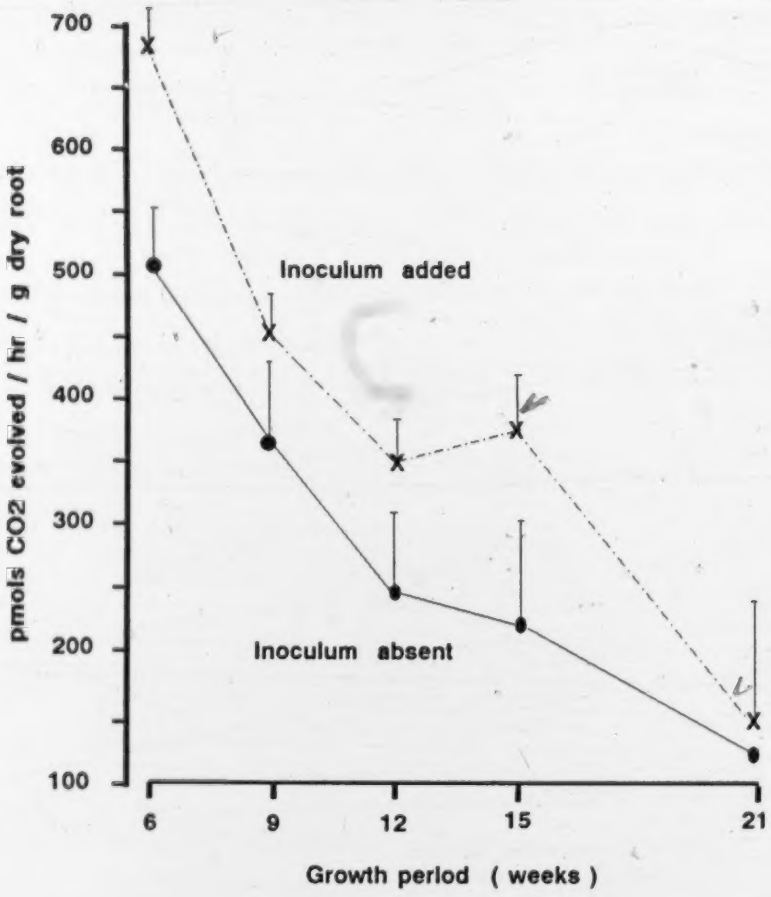

Figure 5.1. ODC activity of pine roots within 21 weeks of planting. Seedlings grown with or without the addition of H. crustuliniforme mycelia. Bars represent standard errors of means.

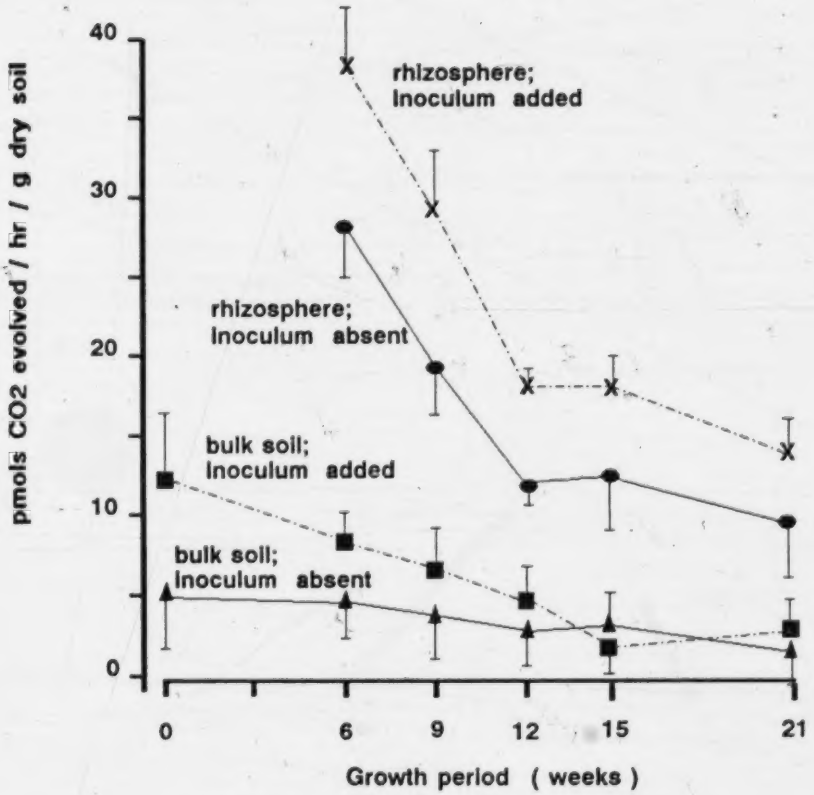

Figure 5.2. ODC activity of bulk soil and rhizosphere of pine roots within 21 weeks of planting. Seedlings were grown with or without the addition of H. crustuliniforme mycelia. Bars represent standard errors of means.

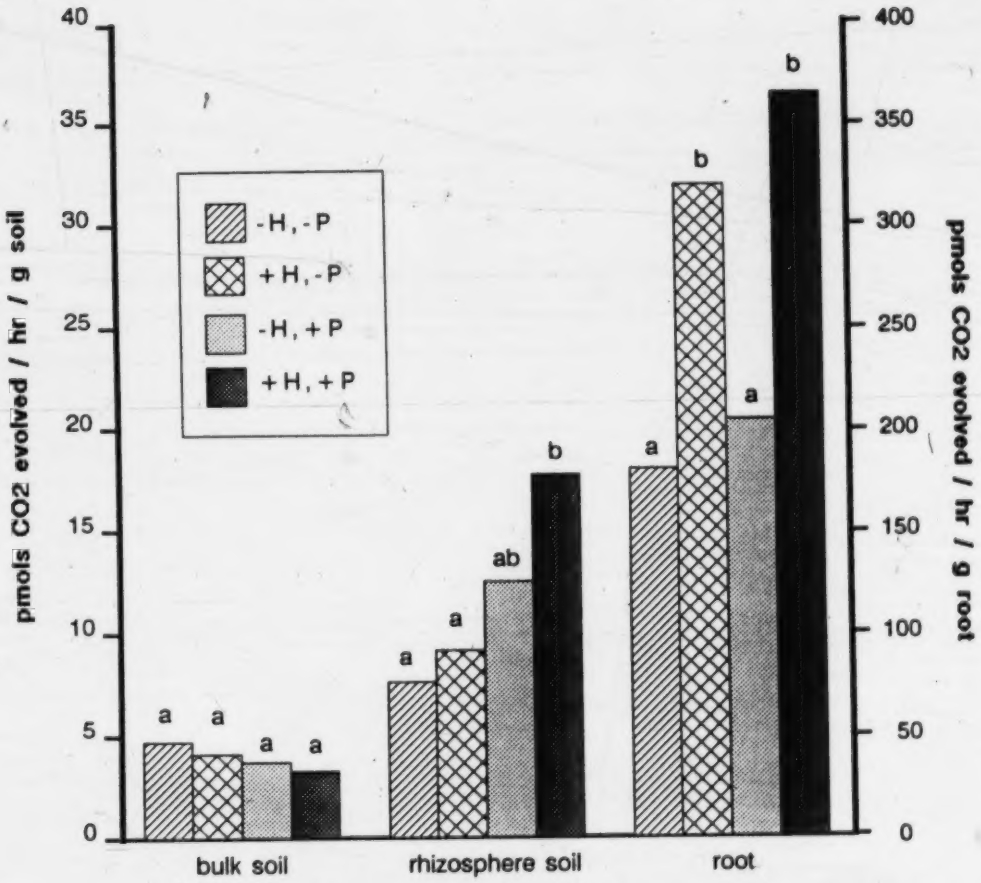

Figure 5.3. ODC activity of bulk soil, rhizosphere soil and 15-week old pine roots grown with or without H. crustuliniforme inoculation or P fertilization. Within each sampled component, treatment means not followed by the same letter are significantly different (LSD; P < 0.05).

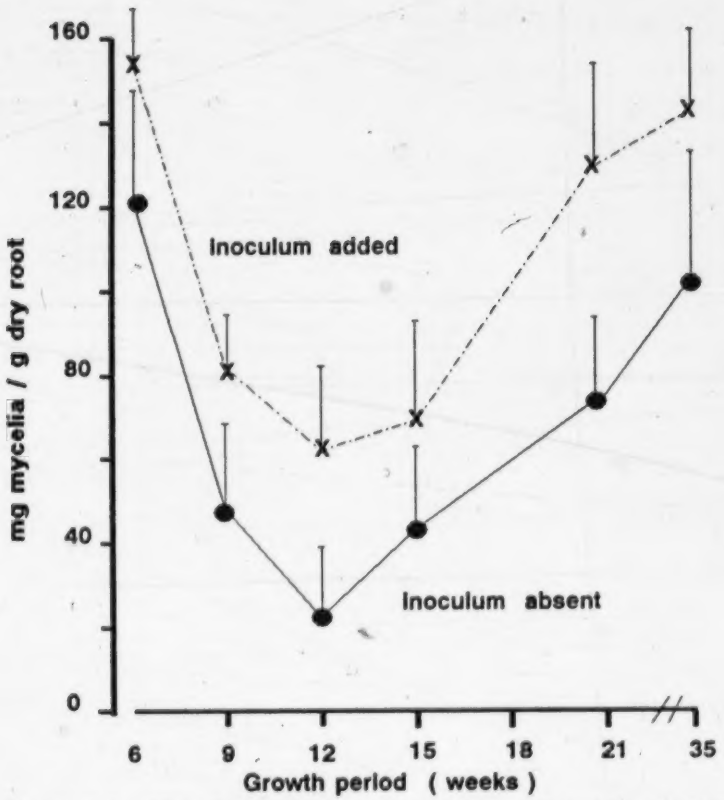

Figure 5.4. Mycelia content of roots within 35 weeks of planting. Seedlings grown with or without the addition of H. crustuliniforme mycelia. Bars represent standard errors of means.

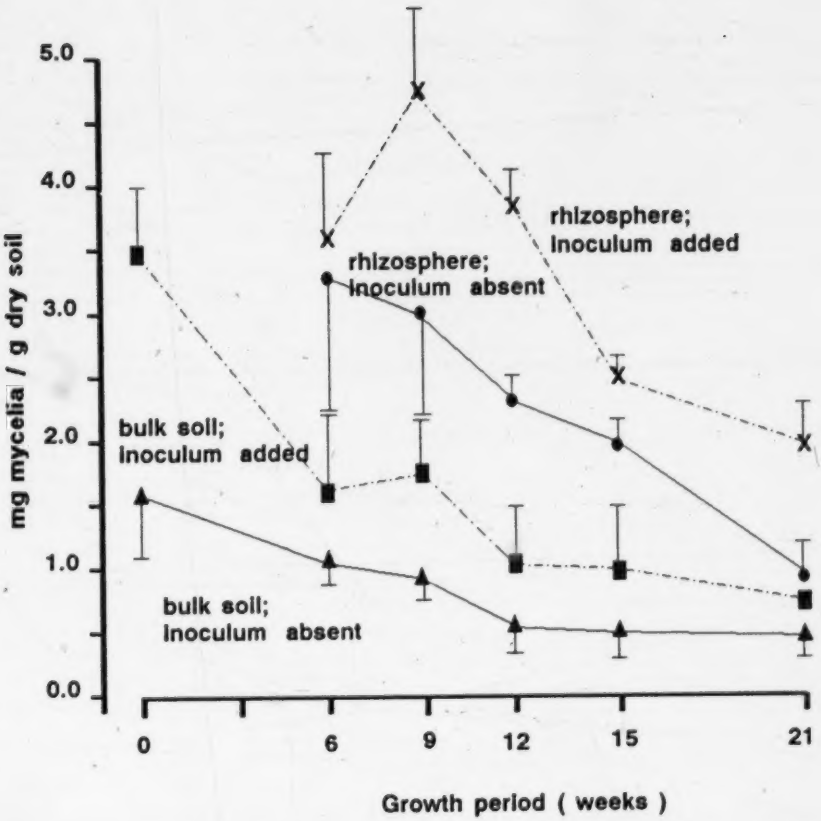

Figure 5.5. Mycelia content of bulk soil and rhizosphere soil of pine roots within 21 weeks of planting. Seedlings were grown with or without addition of H. crustuliniforme mycelia. Bars represent standard errors of means.

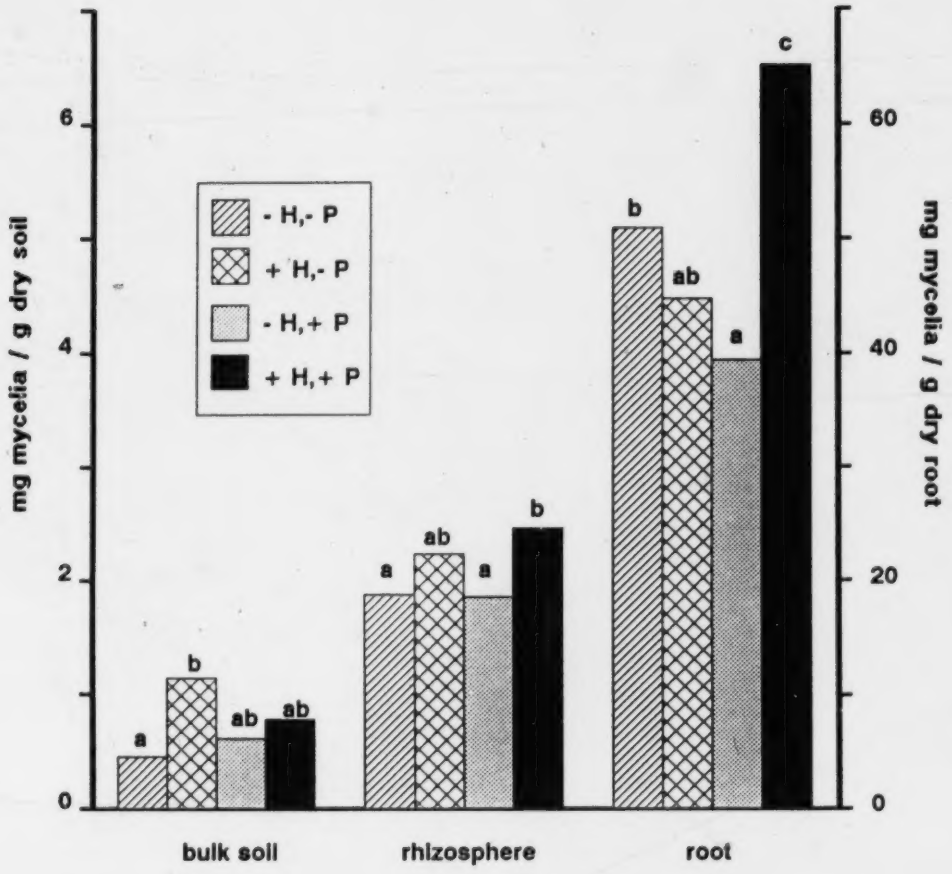

Figure 5.6. Mycelia content of bulk soil, rhizosphere soil and 15-week old pine roots grown with or without H. crustuliniforme inoculation or inorganic P fertilization. Within components, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

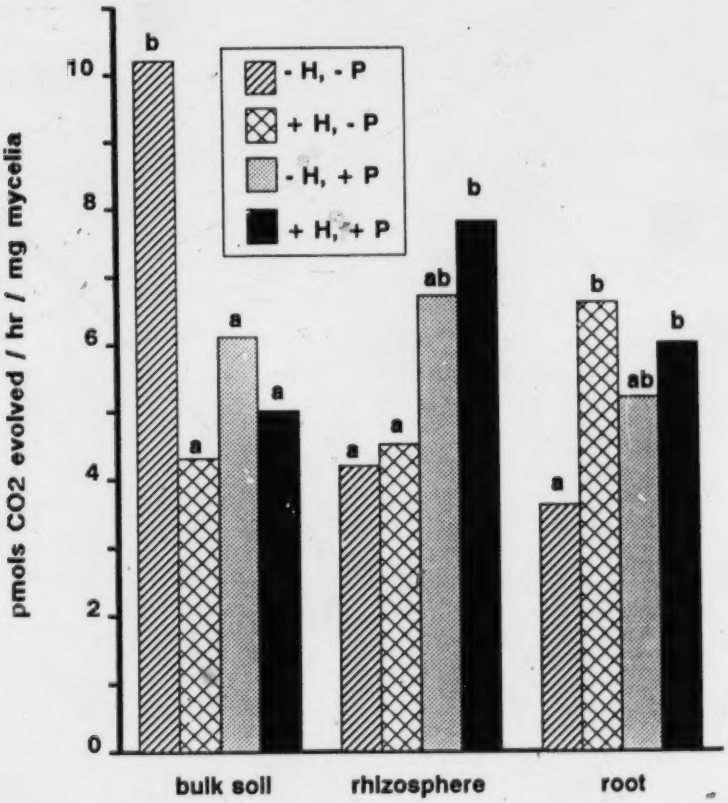

Figure 5.7. ODC activity per mg mycelia in bulk soil, rhizosphere soil and 15-week old pine roots grown with or without H. crustuliniforme inoculation or P fertilization. Within components, treatment means not followed by the same letter are significantly different (LSD; $P < 0.05$).

CHAPTER 6
SYNTHESIS

In previous chapters, I reported on analyses of ergosterol, fungus membrane component and activities of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), enzymes involved in macromolecule synthesis, performed on pure-cultured fungi, soil and mycorrhizal root-soil systems. The intent was to examine the utility of these assays in systems of increasing biological complexity, and under conditions of varying environment and fungal ontogeny. The research was logically but not chronologically organized to describe limitations and potential uses of the assays in 1) pure cultures of an ectomycorrhizal fungus; 2) soil as a habitat for the fungus; and 3) roots and soil as fungal habitats.

Assays were sensitive to changes in the metabolic state of fungal colonies (Chapter 2) and in particular, were affected by mycelia ontogeny. Ergosterol concentration decreased up to 30% and enzyme activities up to 6 fold as mycelia matured or colonies aged, presumably because of vacuolation of hyphae in the filamentous form (Toledo *et al.* 1986). For colonies greater than 300 mg dry mass where a range of maturation states could be expected, coefficients of variability were: 1) 6 to 13% for ergosterol; 2) 22 to 93% for ADC; and 3) 4 to 8% for ODC. Use of ADC activity in determinations of metabolic activity in systems dominated by *H. crustulniforme* are questionable, owing to high variability in ADC activities and the bias towards ODC production, which was 21 to 30 times higher than ADC in pure culture. Ornithine decarboxylase is the preferred polyamine biosynthetic enzyme in many microorganisms (Cohen 1971) and thus, its use in other microbiological systems should be explored.

I concluded that ergosterol was a sensitive indicator of overall mass and was able to describe a relationship between fungal mass and ergosterol mass based on colonies ranging in size from 10 to 700 mg dry mass. Mass of filamentous fungi may be estimated by the regression equation, $Y = -5.46 * 0.38(X)$, where Y = mycelia mass (mg) and X = ergosterol mass (μg). This equation accounted for 77% of the variability in colony mass. I caution against the application of this equation in environments where a variety of fungal structures are present. Spores and other resting bodies may have different sterol contents (Hegnauer and Hohl 1978).

The weak correlation (r=0.15) between ergosterol and chitin contents of roots warrants further examination. The two components differ in cytosolic significance (Elliott 1977) and rates of synthesis dependent upon the environment (Paustian and Schnurer 1987). Therefore, the weak correlation observed here may be attributed to physiologic and morphologic differences among extramatrical and intramatrical mycelia. Salmanowicz and Nylund (1988) observed that ergosterol content of *P. sylvestris* roots varied while percent mycorrhizal root tips did not. This suggests that ergosterol–based estimates of intramatrical mycelial mass may be more informative.

Future attempts should be made to determine correlations between root ergosterol and other biochemical indices. Determination of fluorescein diacetate–active hyphae (Soderstrom 1977) would be appropriate to distinguish active hyphae from inactive. Would this assay yield equal estimates of root infection when compared to the ergosterol assay which appeared to be an index of cumulative mass? France *et al.* (1985) observed that not all short roots are active. By the application of FDA and ergosterol analyses, spatial and temporal maturation trends within mycorrhizal roots may be revealed.

Although possible variations in morphology may confound fungal ergosterol concentration, use of the regression equation revealed temporal changes in mycelia mass in pine roots (Chapter 5), in addition to influences of soil P fertilization and temporal dynamics of intramatrical fungal mass (Chapter 4). Addition of mycorrhizal inoculum increased root mycelia concentration and ODC activity up to 2 fold but also decreased root mass by up to 60% within 21 weeks of planting. The apparent detrimental influences of the mycorrhizal inoculum were time dependent, as inoculated seedlings had 1.7 times greater root mass and 1.3 times greater shoot mass than noninoculated seedlings at 35 weeks. Using ergosterol analyses, it was possible to detect establishment of fungi in roots within 12 weeks. Quantity of infection remained low (less than 4 mg g^{-1} root) throughout this early phase of seedling establishment and then increased to a maximum of 160 mg g^{-1} root at 35 weeks.

Salmanowicz and Nylund (1988) stated that ergosterol is an accurate index of mycorrhizal infection and my research supports this. I further conclude that the assay is sufficiently sensitive to detect changes in fungal mass over time and as a function of soil fertility regime. Application of ergosterol analyses to mycelia mass estimates assumed that the ergosterol content of *H. crustuliniforme* colonies in complex soil and root systems was to equivalent to that of colonies grown on complete Melin Norkran medium. Salmonowicz and Nylund (1988) stated that, for *Laccaria laccata* mycorrhiza, Hartig net and mantle mycelia were of similar ergosterol content to that in liquid culture. Similar relationships need to be verified for *H. crustuliniforme*.

Temporal changes in root:fungal relationships were discernable using the biochemical indices. Further research should be directed to descriptions of mycorrhization and factors influencing the rate and extent of infection. Brown and Sinclair (1981) reported establishment of *Laccaria laccata* on *Pseudotsuga menziesii*

within 2 weeks of radicle emergence, while Chilvers and Gust (1982) observed relatively few mycorrhizal roots in a *Pisolithus tinctorius:Eucalyptus st-johnii* association. In both studies, colonization was measured as epirhizal mycelium. With ergosterol analyses, an opportunity exists to measure intramatrical mycelial establishment in addition to that of the sheath.

Sensitivity of ergosterol concentration to extremes of inorganic-P and organic-C was noted and thus, ergosterol as a biochemical marker is limited by developmental changes and environmental influences similar to the chitin assay (Sharma *et al.* 1977). Nevertheless, the chitin assay has been used extensively for quantifying fungal invasion in plant tissues (Whipps *et al.* 1980). I recommend use of the ergosterol assay in soil research because of low interferences and background values. The environment-induced variations described in Chapter 2 suggest that more research on ergosterol variations is needed.

Activity of ADC was generally low and variable in pure cultured *H. crustuliniforme*. Detectability of ADC may be a limitation in its application to soil. Also, ADC activity appeared to persist in the incubated loamy sand and therefore may not be as desirable as an index of instantaneous activity in soil. Ratios of ODC:ADC activity were not constant during colony growth; the induction or control of ADC and ODC enzyme activities in this fungus were distinct. In particular, ADC activity appeared to decline to a greater degree than ODC in mature colony regions. Induction of these enzymes may be governed at the protein synthesis level (Fuller *et al.* 1978), but it is not possible to suggest adaptive strategies for the maintenance of ODC activities in old mycelia. Further studies of ontological and environmental influences on fungal growth are necessary in this area. In particular, the involvement of polyamines in macromolecule synthesis in fungi has not been clarified.

Recovery of ergosterol and activities of polyamine biosynthesis enzymes from soil was successful (Chapter 3). Mycelia ergosterol could be quantitatively recovered from moist soils only; soil drying likely allowed adsorption of the sterol onto colloidal materials. Ergosterol added as an indigenous component of mycelial tissue was metabolized more rapidly than ergosterol added in a pure form. From monitoring the fate of mycelial ergosterol, I concluded that ergosterol extracted from soil is biontic; less than 15% of the ergosterol added as mycelial tissue reamined as extractable ergosterol after 21 days. Lack of persistence of mycelial ergosterol is a desired attribute for the biochemical index. A compound that tended to accumulate in the substrate would not be desired as a mass index for organisms (Seitz *et al.* 1979). Enzyme activities varied in soil during the incubation period, reflecting early increases in biotic activity, Soil biota generally expressed increases in ODC activity to greater degrees than for ADC. Such a bias in microorganisms has been reported (Cohen 1971). There is an uncertainty about the significance of changes in ODC:ADC activity ratios over time; this may represent a future research avenue of soil biology.

The ergosterol assay was less laborious and detected changes in intramatrical mycelia content upon fertilization of a soil. Thus, it was used in an experiment wherein mycelia were added to a loamy sand along with roots. Ergosterol analyses expressed on a root mass or seedling basis revealed the rates at which mycorrhization may proceed. Infection was observed by 6 weeks, but rapid proliferation of mycelia did not occur until the passage of approximately three months. Thus, the benefit of the fungus to the plant was variable over time.

Hormonal relations and P uptake kinetics are among many key attributes of the mycorrhizal association governing its benefits to plant growth and source:sink relations (Smith 1980). The expression of such interactions may also be time-dependent. Thus, I suggest that this temporal aspect to the nature of

symbiosis be used as a template to study other physiological aspects of the symbiotic relationships among fungi and roots. Polyamine biosynthesis potential and ergosterol content differed between rhizosphere and non-rhizosphere soils, indicating community composition and/or activity differences. The assays performed here may be applied in conjunction with other enzymological or ennumerative methods to further clarfy the extent to which mycorrhization affects rhizosphere soil and non-rhizosphere soil ecology.

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APPENDIX A

Development of Biochemical Methods

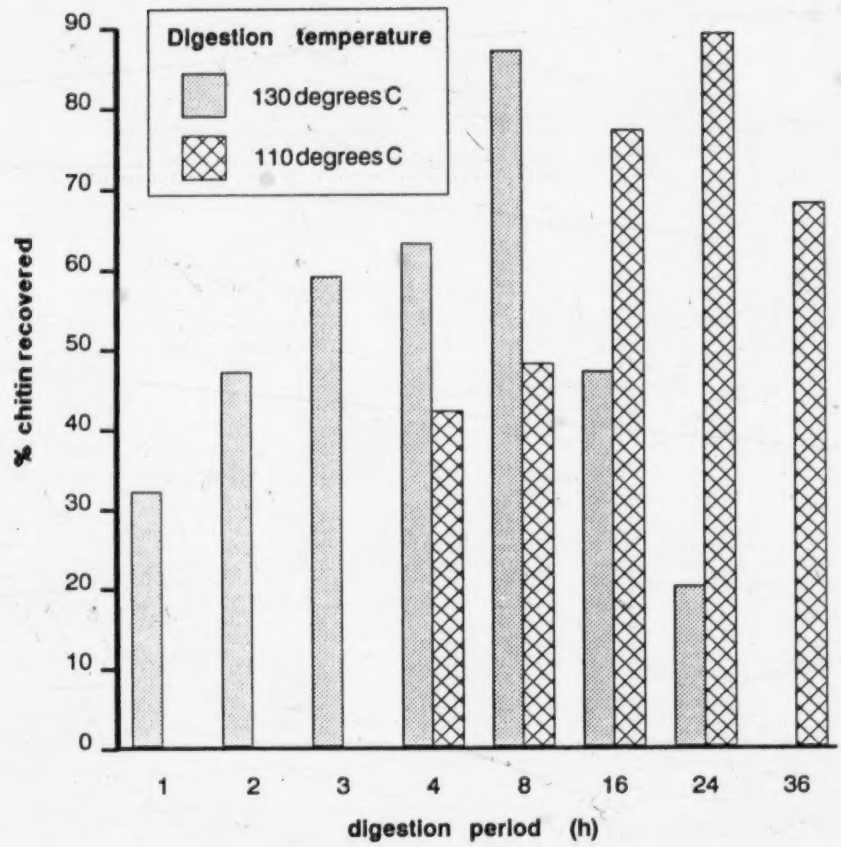

Figure A.1. Recovery of chitin added to mycelia using different KOH digestion times and sand bath temperatures.

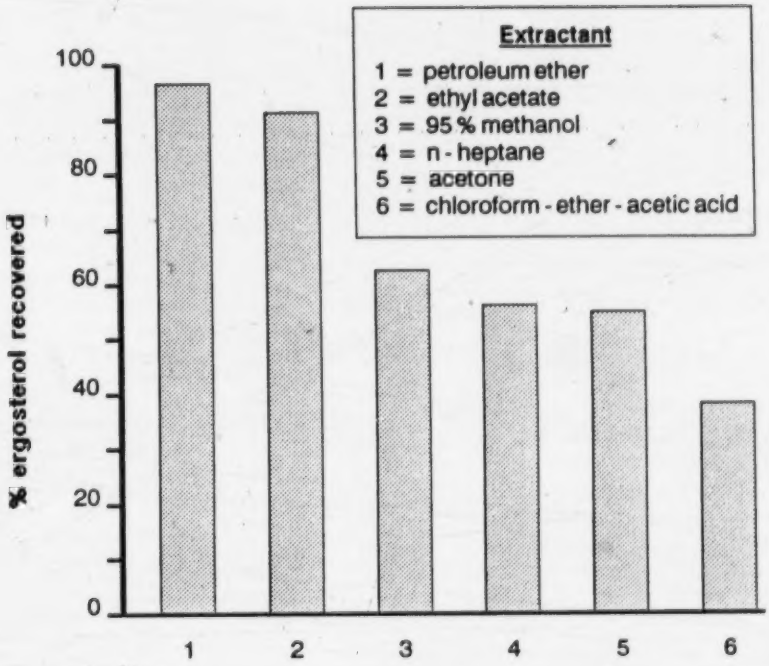

Figure A.2. Recovery of ergosterol added to moist soil using different extractants

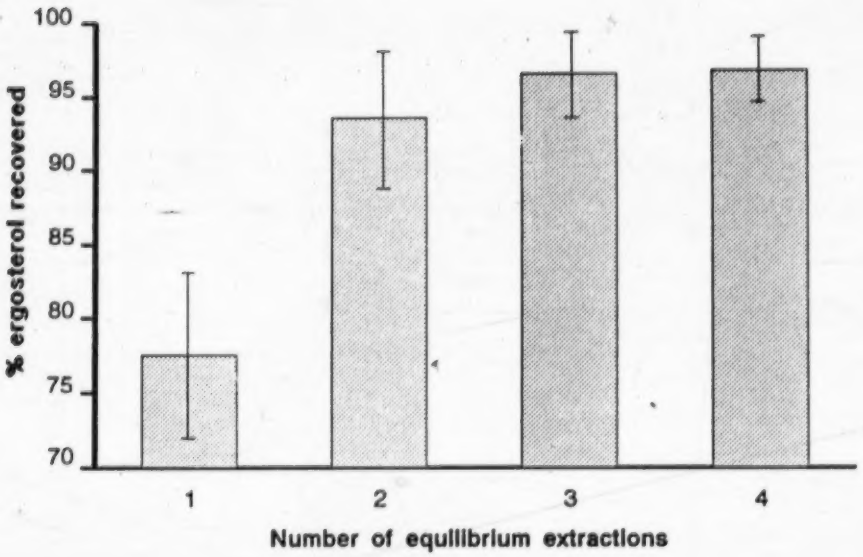

Figure A.3. Recovery of ergosterol added to mycelia using different numbers of equilibrium extractions. Bars represent 2x standard deviations of means.